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The influence of signaling molecules on biofilm formation and antimicrobial resistance

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Dissertation for Master degree in Bioengineering

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Good judgement comes from experience, and a lot of that comes from bad judgement.

Will Rogers

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ABSTRACT

The increased resistance of bacteria to conventional antibiotics is a serious public health problem that is worsened when microorganisms form biofilms. This has led to an increasing interest in the development of new strategies that can provide sustainable and long-term effectiveness against both planktonic and sessile bacteria. Newer strategies have sought to target components of bacteria that are responsible for pathogenesis rather than targeting components that are essential for growth. In this context, quorum sensing (QS) is considered one of the most promising targets for antivirulence therapies. QS is an important regulatory mechanism in biofilm formation and differentiation. So, the disruption of QS signaling pathways can affect biofilm development and make bacteria more susceptible to antimicrobials. Currently, plants have been recognized as a source of unexplored chemical structures with high therapeutic potential. These organisms synthesize a broad array of secondary metabolites (phytochemicals), known for their several biological properties.

In this context, the main aim of this study was to evaluate the potential of fourteen phytochemicals (caffeic acid phenethyl ester (CAPE), rosmarinic acid (RA), sinapic acid (SA), syringic acid (SYR), berberine (BE), piperine (PIPE), piperic acid (PIAC), myrcene (MYR), ocimene (OCI), citronellal (CITA), citronellol (CITO), (-)-carveol (CARV), 2-phenylethyl isothiocyanate (PEITC) and (Z)-2-bromo-5-(2-bromo-2-nitrovinyl)furan (FUR)) and five CAPE derivatives (CABn ((*E*)-benzyl 3-(3,4-dihydroxyphenyl)acrylate), NBn ((*E*)-benzyl 3-(3,4-dihydroxy-5-nitrophenyl)acrylate), CNBn ((*E*)-benzyl 2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)acrylate), NCAPE ((*E*)-phenethyl 3-(3,4-dihydroxy-5-nitrophenyl)acrylate) and CNCAPE ((*E*)-phenethyl 2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)acrylate)) to interfere with distinct *N*-acyl homoserine lactone (AHL)-based QS systems of two Gram-negative bacteria, *Chromobacterium violaceum* and *Pseudomonas aeruginosa*.

The QS inhibition (QSI) activity of selected phytochemicals and/or derivatives was evaluated using the bacterial model *C. violaceum* CV12472 in a disc diffusion assay based on the violacein production (QS-dependent phenotype) followed by the violet pigment extraction and quantification. Moreover, the mechanisms of QSI based on the modulation of AHLs activity and synthesis by the selected compounds were also investigated.

In this context, CABn, PIAC, CITO, CARV and FUR demonstrated a capacity to inhibit QS. Moreover, it was found that CITO and CARV were able to interfere with this cell-to-cell communication process by modulation of AHL synthesis.

In addition to *C. violaceum*, the ability of the selected compounds to interfere with the QS response of opportunistic pathogen *P. aeruginosa* was also evaluated. This was achieved by a high-throughput QSI screening system based on the co-cultivation of *P. aeruginosa* PA14 wild-

type strain with the biosensor *P. aeruginosa* PA14-R3. After the initial QSI screening, the effect of QS inhibitors on PA14 wild-type initial cell adhesion/biofilm development, as well as on the susceptibility of PA14 wild-type biofilms to the antibiotic ciprofloxacin was accessed. For this, a microtiter plate assay was carried out followed by mass and viability quantification of adhered and biofilm cells using crystal violet (CV) and alamar blue stainings, respectively. Further on, the influence of QS inhibitors on the structure of biofilm was studied by epifluorescence microscopy observations using 4',6-diamidino-2-phenylindole (DAPI) staining. Finally, the effect of QS inhibitors on the inhibition of virulence factors production controlled by QS namely pyocyanin, gelatinase, protease and siderophores, was studied.

In this context, RA, SA, SYR, BE, MYR, OCI, CITA, CITO, CARV and FUR were able to interfere with the *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL)-dependent QS system of *P. aeruginosa* by a decrease on bioluminescence with respect to PA14-R3. Adhesion/biofilm studies regarding PA14 showed that RA was able to interfere with initial cell adhesion, as well as SA, SYR and FUR. In addition to cell adhesion, SA, SYR and FUR were also able to interfere with biofilm formation. These last compounds also lead to an increase in PA14 biolfims' susceptibility to ciprofloxacin. Interestingly, even though CITO and PEITC did not affect either cell adhesion or biofilm formation, they also increased biofilm susceptibility to antibiotic. Epifluorescence microscopic analysis showed the ability of all the tested compounds to interfere with extracellular polymeric substance (EPS) production. The results of virulence factors' assays highlighted the potential of FUR to inhibit all the virulence factors tested. Additionally, OCI was found to decrease gelatinase production, whereas PEITC and RA demonstrated potential to inhibit protease production. Finally, BE exhibited a capacity to interfere with siderophores production.

Despite all the promising results obtained with the present study, clinical approval of QS inhibitors demands cytotoxicity studies in order to ascertain their potential use as safe antipathogenic/antivirulent compounds, which sometimes constitutes the ultimate obstacle towards their entry in the market. This can be overcome by searching for anti-QS activity among the thousands of drugs already approved for clinical use. In this context, the present study highlights the potential of FUR, an antibiotic sold under the name of Furvina® which was found to affect QS-dependent phenotypes.

In conclusion, the overall results obtained in this thesis emphasizes the potential of the selected phytochemicals and derivatives as emergent products to inhibit QS-related mechanisms and prevent biofilm formation of important human pathogenic bacteria, being considered an attractive alternative and/or complement to conventional antimicrobials in the treatment of *P. aeruginosa* infections. In addition, some of the phytochemicals studied seemed to be also interesting virulence attenuators.

RESUMO

A crescente resistência de bactérias a antibióticos convencionais é considerada um problema sério de saúde pública que é agravado quando os microrganismos se organizam em biofilmes. Estes factos têm levado a um interesse cada vez maior em desenvolver novas estratégias que possam fornecer um tratamento mais eficaz e a longo-prazo contra bactérias quer em estado plantónico quer em biofilmes. Recentemente têm sido implementadas estratégias inovadoras com o intuito de interferir com componentes de bactérias responsáveis pela sua patogenicidade ao invés de componentes essenciais ao seu crescimento. Neste contexto, o *quorum sensing* (QS) é considerado um dos alvos mais promissores para terapias antivirulentas. O QS é um mecanismo de regulação de extrema importância na formação e diferenciação de biofilmes. Assim sendo, a disrupção de vias sinalizadoras do QS pode afetar o desenvolvimento de biofilmes e tornar as bactérias mais suscetíveis a agentes antimicrobianos. Atualmente, as plantas são fonte de uma riqueza enorme de estruturas químicas inexploradas com alto potencial terapêutico. Estes organismos sintetizam um vasto conjunto de metabolitos secundários (fitoquímicos), também dotados de um conjunto amplo de propriedades biológicas.

Neste sentido, o principal objectivo deste trabalho foi avaliar o potencial de catorze fitoquímicos (éster fenetil do ácido cafeico (CAPE), ácido rosmarínico (RA), ácido sinápico (SA), ácido siríngico (SYR), berberina (BE), piperina (PIPE), ácido pipérico (PIAC), mirceno (MYR), ocimeno (OCI), citronelal (CITA), citronelol (CITO), (-)-carveol (CARV), 2-feniletil isotiocianato (PEITC) e (Z)-2-bromo-5-(2-bromo-2-nitrovinil)furano (FUR)) e de cinco derivados do éster fenetil do ácido cafeico (CABn ((*E*)-benzil 3-(3,4-dihidroxifenil)acrilato), NBn ((*E*)-benzil 3-(3,4-dihidroxi-5-nitrofenil)acrilato), CNBn ((*E*)-benzil 2-ciano-3-(3,4-dihidroxi-5-nitrofenil)acrilato), NCAPE ((*E*)-fenetil 3-(3,4-dihidroxi-5-nitrofenil)acrilato) e CNCAPE ((*E*)-fenetil 2-ciano-3-(3,4-dihidroxi-5-nitrofenil)acrilato)) em interferir com sistemas diferentes de QS baseados em N-acyl homoserina lactonas (AHLs) de duas bactérias Gram-negativas, *Chromobacterium violaceum* e *Pseudomonas aeruginosa*.

A atividade inibitória do QS por parte dos fitoquímicos e/ou derivados selecionados foi avaliada usando, para tal, o modelo bacteriano *C. violaceum* CV12472 num ensaio de disco-difusão baseado na produção de um pigmento roxo denominado violaceína. De seguida, procedeu-se à extração do pigmento e quantificação do mesmo. Os mecanismos de inibição do QS baseados na modulação da atividade e síntese das AHLs foram também determinados.

Assim sendo, os compostos CABn, PIAC, CITO, CARV e FUR demonstraram capacidade para inibir o QS. Adicionalmente, os compostos CITO e CARV foram capazes de interferir com o mecanismo do QS através da modulação da síntese de AHLs.

Para além do *C. violaceum*, a capacidade dos fitoquímicos selecionados para o estudo em questão em interferir com a resposta do QS da bactéria patogénica oportunista *P. aeruginosa* foi também avaliada. Tal foi alcançado a partir do cocultivo da estirpe selvagem *P. aeruginosa* PA14 com o biosensor *P. aeruginosa* PA14-R3. Após um *screening* inicial, o efeito dos inibidores do QS na adesão inicial/desenvolvimento de biofilmes, assim como na suscetibilidade de biofilmes da estirpe selvagem

PA14 ao antibiótico ciprofloxacina, foi testado. Foram levados a cabo ensaios de microplacas seguidos da quantificação da biomassa e da viabilidade celular das células aderidas (2 h) e em biofilme (24 h) usando cristal violeta e *alamar blue*, respetivamente. De seguida foi estudada a influência dos inibidores do QS na estrutura do biofilme. Deste modo, observações ao microscópio de epifluorescência usando o corante 4',6-diamidino-2-fenilindol (DAPI) foram levadas a cabo. Por último, foi estudada a influência dos inibidores do QS na produção de fatores de virulência, tais como a piocianina, gelatinase, protease e sideróforos.

Os compostos RA, SA, SYR, BE, MYR, OCI, CITA, CITO, CARV e FUR foram capazes de interferir com o mecanismo do QS da *P. aeruginosa*, ao nível da molécula sinalizadora *N*-(3-oxododecanoil)-L-homoserina lactona (3-oxo-C12-HSL), através de uma diminuição da bioluminescência emitida pela estirpe PA14-R3. Estudos de adesão celular/desenvolvimento de biofilmes relativamente à estirpe selvagem PA14 demonstraram que o composto RA é capaz de interferir com a adesão inicial, assim como os compostos SA, SYR e FUR. Estes últimos três compostos demonstraram ainda potencial para reduzir a formação de biofilmes e para aumentar a suscetibilidade de biofilmes de *P. aeruginosa* PA14 à ciprofloxacina. Curiosamente, embora os compostos CITO e PEITC não tenham afetado nem a adesão celular nem o desenvolvimento de biofilmes, eles também potenciaram a ação do antibiótico em estudo. A análise microscópica de epifluorescência da estrutura do biofilme permitiu verificar a inibição da produção de EPS. Os ensaios dos fatores de virulência evidenciaram o potencial do composto FUR em inibir todos os fatores de virulência testados. Para além disso, o composto OCI diminuiu a produção de gelatinase, enquanto os compostos PEITC e RA demonstraram capacidade para inibir a produção de proteases. Finalmente, o composto BE interferiu com a produção de sideróforos.

Apesar dos resultados promissores obtidos com este estudo, é necessário realizar ensaios de citotoxicidade para os inibidores do QS eventualmente poderem ser aprovados para uso clínico, o que muitas vezes é considerado o fator decisivo para a entrada do composto no mercado. No entanto, este problema poderá ser ultrapassado se algum destes compostos for já aprovado clinicamente. Neste contexto, este estudo realça o potential do composto FUR, um antibiótico vendido sob o nome de Furvina® em afetar fenótipos intimamente ligados ao QS.

Em suma, os resultados obtidos nesta tese enfatizam o potencial de fitoquímicos/derivados como produtos emergentes para inibição de mecanismos dependentes do QS e para prevenção da formação de biofilmes de importantes bactérias patogénicas humanas, sendo considerados uma alternativa atrativa e/ou um complemento ao tratamento com agentes antimicrobianos de infeções de *P. aeruginosa*.

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GLOSSARY

Abbreviations

3-oxo-C12-HSL	*N*-(3-oxododecanoyl)-L-homoserine lactone
AHL	*N*-Acyl homoserine lactone
AAM	Anacardic acid mixture
ABA	Alkaloid-based analogues
AI	Autoinducer
AM	Antimicrobial
BE	Berberine
CAS	Chrome azurol S
C4-AHL	*N*-butanoyl-L-homoserine lactone
C4-HSL	*N*-butyryl-L-homoserine lactone
C6-AHL	*N*-hexanoyl-L-homoserine lactone
CABn	(*E*)-benzyl 3-(3,4-dihydroxyphenyl)acrylate
CAPE	Caffeic acid phenethyl ester
CARV	(-)-Carveol
CNBn	(*E*)-benzyl 2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)acrylate
CNCAPE	(*E*)-phenethyl 2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)acrylate
CITA	Citronellal
CITO	Citronellol
CFU	Colony forming units
CV	Crystal violet
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DSF	Diffusible signal factor
DWDS	Drinking water distribution systems
EO	Essential oil
EPS	Extracellular polymeric susbtances
FUR	(Z)-2-bromo-5-(2-bromo-2-nitrovinyl)furan
FRCD	Furan ring-containing derivative
GHP	Glucosinolate hydrolysis product
GLS	Glucosinolate

ITC	Isothiocyanate
LB	Luria-Bertani
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
MBC	Minimum bactericidal concentration
MIC	Mininum inhibitory concentration
N/A	Not applicable
NA	No activity
NCAPE	(*E*)-phenethyl 3-(3,4-dihydroxy-5-nitrophenyl)acrylate
OCI	Ocimene
PCA	Plate count agar
PIAC	Piperic acid
PIPE	Piperine
PP	Pigment production
PQS	*Pseudomonas* quinolone signal
PS	Polystyrene
QS	Quorum sensing
QSI	Quorum sensing inhibition
RA	Rosmarinic acid
RT	Room temperature
SA	Sinapic acid
SYR	Syringic acid
USA	United States of America
WHO	World Health Organization

Indexes and parameters

A_x	Absorbance at x nm
d_1	Diameter of bacterial growth inhibition
d_2	Total diameter
LCPS	Light counts per second

CHAPTER 1

Work outline

1.1 Background

Over the last century, excessive and indiscriminate use of antibiotics has led to the emergence of multidrug-resistant bacteria. This is considered a serious public health problem that is worsened when microorganisms form biofilms (Kalia 2013). Therefore, there is an urgent need for the development of therapies that can provide sustainable and long-term effectiveness against pathogenic bacteria (LaSarre and Federle 2013). QS is an important mechanism that regulates bacterial behavior within biofilms. In addition to its role in biofilms, QS regulates the expression of genes involved in processes related to survival, virulence, and pathogenicity. So, the disruption of QS signaling pathways can affect biofilm development and make bacteria more susceptible to antimicrobials (Kalia 2013). Phytochemicals (secondary metabolites from plants) have demonstrated distinctive properties that can help to overcome the resistance problem and make them perfect candidates for these much needed therapeutics (Truchado et al. 2015).

1.2 Research objectives

The main objective of this study was to evaluate the potential of selected phytochemicals and its derivatives belonging to chemical classes of phenolics (simple phenolics and phenolic acids), alkaloids (simple alkaloids and alkaloid based analogues (ABA)), terpenoids, glucosinolate hydrolysis products (GHPs) (isothiocyanates - ITCs), and furan ring-containing derivatives (FRCDs) to interfere with distinct AHL-based QS systems of two Gram-negative bacteria, *C. violaceum* and *P. aeruginosa*.

Phenolics, alkaloids, terpenoids, GHPs and FRCDs can be commonly found in diverse dietary products, and their release is related to defense mechanisms against microbial attack. These compounds are among the most important plant secondary metabolites due to their recognized biological properties (table A1 in Appendix). Thus, considering their numerous therapeutic properties, and the fact that these compounds are thought to be an integral part of both human and animal diets, it is important to study their antipathogenic and antibiofilm potential.

C. violaceum, despite its low pathogenicity, is being used as a model organism in diverse studies on Gram-negative bacteria due to its AHL-based QS system and also its QS dependent phenotype (purple pigment production) that can be easily detected visually making it excellent for initial screening. (Chaudhari et al. 2014; Hagiya et al. 2014). Therefore, a disc diffusion assay based on the pigment inhibition of biosensor strain *C. violaceum* CV12472 was performed. The amount of violacein produced after exposure to selected phytochemicals was also quantified. In addition, the mechanisms of QS inhibition (QSI) based on the modulation of AHLs activity and synthesis by the phytochemicals in study were also investigated. To achieve these results, a qualitative agar diffusion double ring assay with two strains, *C. violaceum* CV026 (AHL biosensor) and *C. violaceum* CV31532 (AHL overproducer), was performed.

P. aeruginosa possesses a more complex QS system and it is considered a clinical important opportunistic pathogen involved in many severe infections. Moreover, it is provided with an outstanding capacity to become resistant to multiple drugs. Because *P. aeruginosa* is a good biofilm producer, it is also frequently implicated in infections related to biofilm formation. QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for biofilm formation (Imperi et al. 2013). Thus, as QS is an important phenomenon that is related with different steps of biofilm formation and differentiation, it is also an objective of this work to evaluate if the phytochemicals with QSI activity are likely to influence biofilm development and aspects related with its formation, such as initial cell adhesion, and structure. Additionally, susceptibility of *P. aeruginosa* biofilms grown in the presence of phytochemicals (identified previously as QS inhibitors) to an antibiotic was also accessed. To evaluate the potential of phytochemicals to interfere with the QS system of this bacterium, a high-throughput QSI screening system based on the co-cultivation of *P. aeruginosa* PA14 wild-type strain with the biosensor *P. aeruginosa* PA14-R3 was carried out. To study the effect of QS inhibitors on PA14 wild-type initial cell adhesion/ biofilm development, a microtiter plate assay was carried out followed by quantification of the mass and viability of adhered and biofilm cells using crystal violet (CV) and alamar blue, respectively. Further on, the influence of QSI inhibitors on the structure of biofilms was studied by microscopic observations using the specific staining 4',6-diamidino-2-phenylindole (DAPI). The susceptibility of PA14 wild-type biofilms (grown in the presence of QS inhibitors) to ciprofloxacin was performed using a microtiter plate assay, followed by mass and viability quantification of biofilm cells using CV and alamar blue, respectively. Even though QS controls genes responsible for biofilm formation, this mechanism is also responsible for the expression of genes related with the secretion of virulence factors in *P. aeruginosa* (Imperi et al. 2013). Hence, the effect of QS inhibitors on the inhibition of virulence

factors production controlled by QS namely pyocyanin, gelatinase, protease, and siderophores, was too studied.

1.3 Thesis organization

This work is divided in five chapters. Chapter 1 describes the main goals, context and motivations for the development of this study. It is also a guideline on the overall work developed.

Chapter 2 provides a brief review of the literature. In this chapter, the major obstacles towards the treatment of bacterial infections are highlighted. The ability of bacteria to organize themselves in structured communities named biofilms is here described, with particular focus on both the formation process and repercussions in public health. Also in this chapter, the QS phenomenon is exploited and its inhibition is introduced as a promising therapy to make bacteria less pathogenic and biofilms more susceptible to antimicrobials. The importance of natural products from plants as a source of QS inhibitors is presented. The main classes of phytochemicals with recognized antimicrobial activity are also referred in this chapter. The use of phytochemicals as QS inhibitors is also reviewed, considering the existent literature.

Chapters 3 and 4 are both comprised of a very brief introduction to the subject addressed, followed by the implemented methodology and ending with the obtained results and subsequent discussion. Chapter 3 focus on the study of QSI activity of thirteen phytochemicals (CAPE, RA, SA, SYR, BE, PIPE, PIAC, MYR, OCI, CITA, CITO, CARV, and FUR) and five derivatives (CABn, NBn, CNBn, NCAPE and CNCAPE) against *C. violaceum.* Quantitative and qualitative results for the determination of violacein inhibition are here displayed. The mechanisms of QSI based on the modulation of AHLs activity and synthesis by the phytochemicals are also investigated.

In chapter 4 the QSI activity against *P. aeruginosa* by ten (RA, SA, SYR, BE, MYR, OCI, CITA, CITO, CARV, and FUR) out of the thirteen phytochemicals tested in chapter 3 were studied. Moreover, PEITC was added to this study. Sensitivity problems of some methods used in this study and solubility issues of some compounds precluded the used of CAPE, CAPE derivatives, PIPE and PIAC. The ability of the putative QS inhibitors to influence either biofilm development and aspects related with their formation, such as initial cell adhesion, and structure, was evaluated, as well as the susceptibility of biofilms (grown in the presence of the QS inhibitors) to the antibiotic ciprofloxacin. Additionally, the effects of QS inhibitors on virulence factors production controlled by QS were also studied.

Finally, chapter 5 presents an overview of all the developed work, with emphasis on the main conclusions and on the perspectives for further research.

CHAPTER 2

Literature review

2.1 Current barriers in the treatment of bacterial infections

The discovery of antibiotics during the 20th century is considered one of the most important achievements in the history of medicine, bringing relief to humans with regards to a large number of life-threatening and debilitating diseases (Kalia 2013; LaSarre and Federle 2013). Although antibiotics have proven to be powerful drugs for the control of infectious diseases, their extensive and unrestricted use over the last century has imposed selective pressure upon bacteria, leading to the development of resistance. According to the World Health Organization (WHO) antimicrobial resistance is a global problem that is considered the major threat in the treatment of infectious diseases (Abreu et al. 2012). Efforts have been made in order to develop new drugs aimed at thwarting the emergence of antibiotic resistances, such as resistance to vancomycin and to the latest generation of beta-lactams. However, several of these antibacterial compounds have already lost effectiveness against some bacterial strains (LaSarre and Federle 2013). Even more disheartening is the fact that the pharmaceutical companies have slowed dramatically the development of new drugs over the past 10 years, and the ones successfully developed are strictly intended to treat only the most serious infections (Dellit et al. 2007).

Apart from the antibiotic resistance problem, the premise that bacteria in a sessile state (biofilms) are highly resistant to antimicrobials when compared to their planktonic (free-living) counterparts is also considered an obstacle to the treatment of bacterial infections (Lewis 2001). For some bacteria, working together as a group provides a means to build a defense that planktonic cells find impossible to achieve (LaSarre and Federle 2013). Indeed, bacteria in biofilms are more protected against host defenses, and highly tolerant to antimicrobials (100-1000 times more) when compared to their planktonic counterparts (Gilbert et al. 2002).

2.2 Biofilm: an advantageous microbial lifestyle

Bacteria as well as other microorganisms such as protozoa, algae, and fungi are able to colonize and thrive in a variety of different environments by forming biofilms (Galante et al. 2015). A biofilm is defined as a complex microbial community composed by cells irreversibly attached to a substratum, to an interface or to each other, and encased in a matrix of

extracellular polymeric substances (EPS) produced by themselves (Donlan and Costerton 2002). This lifestyle is recognized as the most successful and competitive expression of the prokaryotic genome, with their cells being metabolically more efficient, well protected and resistant to any kind of stress. In sessile state, bacterial cells differ considerably from the planktonic mode of growth in terms of behavior, structure and physiology (Costerton et al. 1995; Stoodley et al. 2002). The modified phenotype presented by biofilm cells is related with their altered growth rate and gene transcription. Typically, most bacterial species alternate between free-living and biofilm states in response to environmental stimuli such as the availability of essential nutrients, bacterial cell density, and cellular stress (Lynch and Robertson 2008).

2.2.1 Biofilm formation

Biofilms can be formed on a wide variety of biotic and abiotic surfaces, such as living tissues, indwelling medical devices, industrial/potable water system piping and natural aquatic systems (Donlan 2002; Percival et al. 2011).

The development of a mature biofilm is a dynamic and multicellular process that encompasses several steps (Figure 1). To begin, the adsorption of layers of macro/micromolecules, namely glycoproteins, polysaccharides and lipids, takes place on the adhesion surface, forming a conditioning film. This surface conditioning step alters the physicochemical characteristics of the interface, such as electrical charge and hydrophobicity, enabling cell attachment (Dunne 2002). Both molecules and cells are transported to the surface by a combination of convection, diffusion and sedimentation events (Busscher and van der Mei 2000; Percival et al. 2011).

The next step usually consists in adhesion of the microorganisms, which can be split into two phases involving reversible and irreversible processes. Reversible adhesion comprises the initial weak bonding of microbial cells to a conditioned surface, whereas irreversible adhesion refers to the permanent attachment of microorganisms (Dunne 2002). Cell surface hydrophobicity and roughness, the presence of extracellular appendages, and mainly the quantity and composition of generated EPS, are considered the main factors with influence in both the rate and degree of microbial adhesion (Donlan 2002; Percival et al. 2011).

Following adhesion of microbial cells to the substratum, formation of microcolonies (cell-to-cell adhesion) takes place. This step involves initial production of EPS matrix and multiplication of the attached organisms and/or attachment of other bacteria to already adhered cells, in a phenomenon named coadhesion which is temperature, pH and ionic strength

dependent. Moreover, it is also in this step that extensive cellular differentiation begins to be observed (Busscher and van der Mei 2000).

During cell growth, the biofilm develops complex three-dimensional structures, providing to their microbial cells niches with different physicochemical conditions. Therefore, cells in different regions of the biofilm will experience distinct patterns of gene expression (Costerton et al. 1999). This process is mediated by production of signaling molecules in a phenomenon named QS (discussed later below) (Davies et al. 1998).

After the full development of a biofilm is accomplished, cells begin to senesce and detach. Cells can detach from biofilm by physical (erosion, shear forces, sloughing, human intervention) or physiological factors (activation of specific enzymes) (Costerton et al. 1995; O'Toole et al. 2000). Nutrient and oxygen depletion, temperature, and pH can also lead to biofilm detachment (Percival et al. 2011).

From an evolutionary standpoint, biofilm detachment is beneficial as it increases the genetic diversity, and the colonization capacity of new niches. However, this process has very important repercussions to public health, namely to the medical sector, increasing the incidence of nosocomial infections (Borges et al. 2015). Although the clinical impact is usually the most relevant, with 80% of the microbial infections associated with this complex mode of life, impacts on industry (e.g. biocorrosion and food spoilage) and environment (e.g. drinking water distribution systems (DWDS)) are also of concern (Adetunji et al. 2014; Tolker-Nielsen 2014; Rozej et al. 2015).

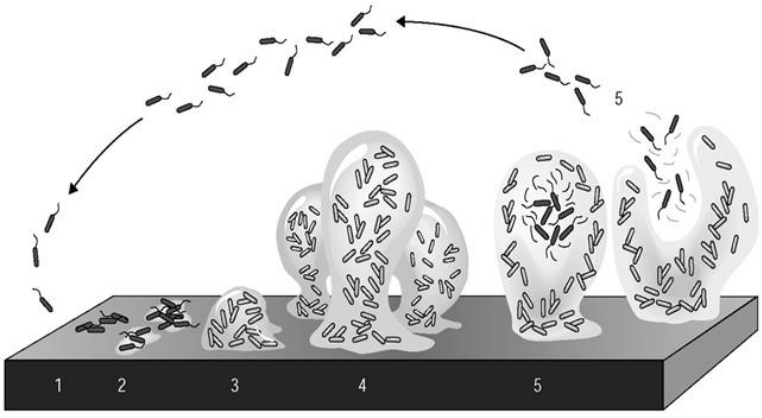


Figure 1. Stages of bacterial biofilm development: (1) the bacterial cells attach reversibly to the surface; (2) the cells attach irreversibly and lose their flagella-driven motility, a step mediated mainly by exopolymeric substances; (3) the first maturation phase is reached, as indicated by early development of biofilm architecture; (4) the second maturation phase is attained with fully mature biofilms, as indicated by the complex biofilm architecture. Cells in different regions of the biofilm will experience distinct patterns of gene expression, regulated by QS; (5) single motile cells disperse from the microcolonies (Sauer 2003).

2.2.2 Biofilm-related problems

In a clinical point of view biofilm formation and persistence has enormous implications for the human health. In fact, biofilms formed by pathogenic bacteria and fungi are associated with a wide range of diseases, from device-related to chronic infections occurring on native tissues (Beloin et al. 2014). For instance, persistence of staphylococcal infections related to the implantation of biomedical devices is due to biofilm formation. In the same way chronic *P. aeruginosa* lung infections in cystic fibrosis patients are caused by biofilm growing mucoid strains (Hoiby et al. 2011). Other pathogenic bacteria include *Clostridium* spp. which causes gangrene, *Enterobacteriacae* (e.g. *Proteus, Klebsiella, Enterobacter, Serratia marcescens*) which colonize sites where the host defenses are compromised, causing serious infections (bacteraemia, lung and peritoneum infections), and *Legionella pneumophila*, which may cause pneumonia through inhalation of aerosols containing contaminated water. Fungi and other parasites, namely, *Candida albicans*, *Aspergillus* spp., *Cryptococcus neoformans*, and *Cryptosporidium* spp. are also responsible for the emergence of infections during extended antibiotic treatment and severe immunosuppression (World Health Organization 2002). The economic burden of these infections is tremendous leading to longer hospital stays, recurrent infection, and increased fatalities in the most recalcitrant cases. In the United States of America (USA), nosocomial infections are caught by 2 million patients every year with 100000 associated deaths, making this type of infection the fourth main cause of death in this country. Regarding the European Union, nosocomial infections affect 3 million patients per year resulting in 50000 deaths (Bryers 2008).

Bacterial biofilms are also a major source of food contamination which can lead to foodborne disease outbreaks. Because the hygiene of the surfaces affects the overall quality and safety of the food product, biofilm is a major problem in the food industry. Numerous studies have shown that *Listeria monocytogenes* adheres and forms biofilm on metal, glass or rubber surfaces and *Escherichia coli* and *Staphylococcus aureus* are also capable of forming biofilms on polypropylene mesh (Adetunji et al. 2014)

Likewise, DWDS are affected by biofilm proliferation, where they assume considerable hygienic operational and economical relevance. The presence of biofilms in these systems affect the microbiological quality of water and may lead to a number of undesirable effects on their organoleptic properties (turbidity, taste, odor and color) (Simões and Simões 2013). Due to the dynamic nature of biofilms, some portions are frequently sloughed of pipe surfaces, constituting a potential source of secondary microbial contamination of water (Rozej et al. 2015). Besides, they can provide a possible habitat for clinically relevant pathogens which are responsible for several waterborne diseases (Huq et al. 2008).

Clearly, biofilm formation is a serious worldwide problem, affecting distinct fields with severe consequences for public health (Beloin et al. 2014). These observations, along with the fact that current antibiotics rely on inhibition of bacterial growth, imposing strong selective pressure on bacteria that can lead to the development of resistance mechanisms, highlight a strong need to develop therapies that can provide sustainable and long-term effectiveness against bacterial pathogens (Landini et al. 2010; LaSarre and Federle 2013). Newer strategies have been focused on targeting bacterial cellular processes responsible for pathogenesis rather than components essential for their growth and thus have garnered the name, antivirulence therapies (LaSarre and Federle 2013).

2.3 Interfering with quorum sensing: an antivirulence therapy

Bacteria commonly benefit from social interactions and intercellular signaling. QS, a form of intercellular communication used by many species of bacteria, is currently considered a hot topic in microbiology and it seems to provide an alternative to the ineffective conventional biofilm control strategies (Simões et al. 2009; Defoirdt et al. 2013). QS relies on the production, release and detection of small signaling molecules called autoinducers (AIs), much like hormones in higher organisms. These molecules are responsible for the initiation of coordinated responses across a population, such as the expression of virulence genes, in a cell density dependent manner (Galloway et al. 2012; LaSarre and Federle 2013). This complex gene regulatory system is comprised of three components: the AI, the gene coding for the AI synthase protein and the gene coding for a response regulator protein (Miller and Bassler 2001). At low densities, bacteria behave as single cellular organisms, producing and secreting normal levels of AIs (Kalia 2013). However, when the cell density starts to increase, the level of released AIs also increases. The signaling molecules begin to accumulate in the surrounding environment and when their extracellular concentration reaches a critical threshold level, a signal transduction cascade is triggered. This mechanism leads to population-wide changes in gene expression and also the initiation of cooperative behaviors that benefit the community as a whole (Whitehead et al. 2001; Daniels et al. 2004; Galloway et al. 2012).

Despite the regulation by QS being extremely conserved in bacteria, its molecular mechanism, as well as the chemical nature of the AIs, differ considerably between Gram-negative and Gram-positive bacteria and depend on the particular species (Miller and Bassler 2001). Several chemical classes of microbial derived signaling molecules were already identified (Figure 2), with the most commonly studied ones belonging to one of the following three categories: AHLs, also referred to as AI-1, used by Gram-negative bacteria; AI peptides

(AIPs), produced by Gram-positive bacteria; and AI-2, a furanosyl borate diester, which is considered a "universal signal" involved in inter-specific communication in both Gram-negative and Gram-positive bacteria (Miller and Bassler 2001; Worthington et al. 2012). Other quorum sensing signals that go beyond these classes have been also described, including *Pseudomonas* quinolone signal (PQS), diffusible signal factor (DSF), and AI-3 (LaSarre and Federle 2013).

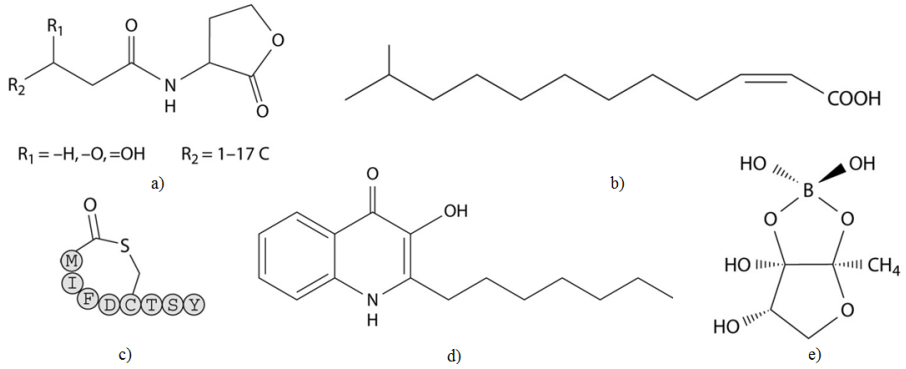


Figure 2. Structure of five AIs: a) AHL; b) DSF from *Xantomonas campestris*; c) AIP (group I) from *S. aureus*; d) PQS from *P. aeruginosa*; e) AI-2 from *Vibrio harveyi* (LaSarre and Federle 2013).

In many cases, the responses prompted by QS signals contribute directly to pathogenesis through the production of virulence determinants, such as toxins and proteases (LaSarre and Federle 2013). In fact, various pathogenic bacteria such as *P. aeruginosa*, *Vibrio* sp., *Burkholderia cepacia* and *Yersinia enterocolitica* employed QS to regulate their virulence and pathogenicity (Khan et al. 2009). Additionally, QS can contribute to behaviors such as biofilm development that enable bacteria to acquire resistance against antimicrobial compounds (e.g. antibiotics and disinfectants) (LaSarre and Federle 2013). Indeed, QS systems are almost always integrated into some processes important to initiate biofilm formation, namely bacterial adhesion (e.g. secretion of adhesins), bacterial motility, formation of microcolonies and EPS production (Vu et al. 2009; Packiavathy et al. 2012; Lee et al. 2013). Hence, if these efforts to coordinate bacteria are blocked, it is theorized that bacteria will lose their ability to mount an organized assault on the host's defense and/or be less able to form organized microbial communities that promote pathogenesis, such as biofilms. One of the keys of success in targeting QS is the evidence that a compound that does not suppress cell growth will not exert selective pressure to develop resistance to that treatment (LaSarre and Federle 2013). In addition, as QS regulates the expression of genes involved in processes related to survival, virulence, and pathogenicity, their inhibition can also improve the therapy with antibiotics, increasing their effectiveness. QSI favors the use of low doses and avoids the indiscriminated use of broad-spectrum antibiotics (Castillo-Juarez et al. 2013).

Even though the process of QS used by a given bacterium is unique in its own way, all QS systems share a circuit composed of signal production, signal accumulation and signal detection mechanisms. Hence, whatever the efforts employed to disrupt this phenomenon, all strategies will be based in inhibiting one of these mechanisms (Kalia 2013; LaSarre and Federle 2013). A wide variety of QS inhibitors have been already discovered, however, they have not been qualified as chemotherapeutic agents due to their high reactivity, toxicity and instability (Khan et al. 2009). The current quest for new antimicrobials is therefore aimed at discovering non-toxic QS inhibitors from natural and sustainable sources (Hentzer and Givskov 2003).

Numerous reviews with respect to important natural products used to treat human diseases have been extensively described. They include compounds derived from microbes (fungi and bacteria), plants, animals and marine sources (Newman and Cragg 2000; Newman and Cragg 2012). In fact, more than 80% of drug substances used nowadays to treat and prevent a wide range of diseases (infectious diseases, neurological diseases, cardiovascular diseases, neoplasia and oncological diseases, immunological, inflammatory and related diseases) are natural products or based on natural scaffolds (Butler 2008; Harvey 2008; Newman and Cragg 2012). Nevertheless, it has been predicted that fairly little of the world's plant biodiversity has been screened for their bioactivity (Harvey 2008). Hence, despite decades of research, all evidences lead to believe that there must remain many interesting natural molecules derived from plants with potential therapeutic application yet to be discovered (Gao et al. 2003; Borges et al. 2015).

2.4 Phytochemicals as a source of new drugs

Since ancient times, plants have been documented for their versatile applications and particularly for their nutritional and medicinal use (Truchado et al. 2015). They have the ability to synthesize a broad range of natural secondary metabolites known as phytochemicals, many of which play a crucial role in plants defense and have evolved to confer selective advantage against microbial attack and even other plants (Dixon 2001). The study of pathways involved in production of phytochemicals and their role in plant defense mechanisms against pathogens has led the scientific community to exploit the biological properties of such compounds (Borges et al. 2015).

As such, many research studies have recognized these secondary metabolites of plants as a large and attractive repertoire of QS inhibitors (Vattem et al. 2007), offering an outstanding chemical diversity with structural complexity and biological activity (Borges et al. 2015). One key advantage of the use of phytochemicals over other molecules is their resemblance to what is

considered the ideal QS inhibitor, which includes being chemically stable, highly effective low-molecular-mass molecules, and harmless for human health. In fact, unlike many antibiotic regimes, the consumption of phytochemicals is rarely associated with any side-effect (Rasmussen and Givskov 2006).

Several studies suggest that phytochemicals may result in a new category of antimicrobial substances with a much broader spectrum and may overcome the issue of developing antibiotic resistance (Mowrey 1990; Vattem and Shetty 2005). In this context, detailed structural analysis of these sources has determined different categories of phytochemicals. (Vattem et al. 2007).

2.4.1 Classes of phytochemicals

The most important groups of phytochemicals can be divided into several categories that include phenolics and polyphenolics, terpenoids and other essential oils components, alkaloids, lectines and peptides, and polyacetylenes (Borges et al. 2015).

Phenolic compounds are considered one of the most diverse groups of phytochemicals (Saleem et al. 2010). They are a large group of aromatic compounds which based solely on their number of phenol subunits can be subdivided into three main categories: phenolic acids, flavonoids and tannins. Phenolic acids constitute one of the major classes of phenolic compounds, with substituted derivatives of hydroxybenzoic and hydroxycinnamic acids as the most predominant ones (Robbins 2003). Flavonoids are seen as one of the largest classes of secondary metabolites from plants (Nijveldt et al. 2001). These compounds share a common structure consisting of 2 aromatic rings that are bound together by 3 carbon atoms that form an oxygenated heterocyclic. Regarding the type of heterocyclic, flavonoids can be divided into flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols (catechins and proanthocyanidins) (Manach et al. 2004). Tannins can be divided into hydrolysable (based on gallic acid moiety) and non-hydrolysable (derived from flavonoid monomers and called proanthocyanidins) tannins (Hassanpour et al. 2011).

Alkaloids are a highly diverse group of heterocyclic nitrogen compounds (Samy and Gopalakrishnakone 2010). Antimicrobial alkaloids are produced by a wide array of plant families, and several of these compounds are reported to own powerful antimicrobial properties that could therefore be a good alternative for current drugs (Saleem et al. 2010).

Plant essential oils (EOs) are volatile aromatic materials enriched with compounds based on an isoprene structure with additional elements such as oxygen named terpenoids (Bakkali et al. 2008); Samy and Gopalakrishnakone 2010). These compounds, also called terpenes,

represent a large and diverse class of naturally occurring compounds found in a variety of natural sources including plants. These compounds are classified according to the number of basic isoprene units. In EOs, terpenoids are present as either monoterpenoids (two units) or sesquiterpenoids (three units), and their derivatives (Wang et al. 2005).

Antimicrobial peptides are short-length peptides (between 15 and 30 amino acids) with microbicidal activity (Wiesner and Vilcinskas 2010). They encompass several protein groups with distinct features, mostly on the total charge of the molecule and on the content of disulphide bonds (Pelegrini et al. 2011). These compounds can be classified into distinct families based on the primary structure, size and cysteine content, namely plant defensins, thionins, snakins, lipid transfer proteins, and hevein-and knottin-like proteins (Broekaert et al. 1997; Pushpanathan et al. 2013; Stotz et al. 2013).

Lectins are an important group of biologically active proteins or glycoproteins which share a common activity but have distinct properties regarding sizes, structures, molecular organization and active sites (Damme et al. 1998; Karnchanatat 2012). In general, there are no common structural features to all lectin families (Karnchanatat 2012).

Polyacetylenes are derivatives of fatty acids that are characterized by one or more acetylenic groups in their structures (Christensen and Brandt 2006). Due to their biological properties, these compounds possess beneficial effects for human health, namely anti-inflammatory, antiallergenic, anticancer, antifungal, and antibacterial activities (Blasa et al. 2010; Kuklev et al. 2013).

Glucosinolates (GLS) are an important group of phytochemicals that can be found in large numbers of edible plants. They are organosulfur compounds that can be grouped into aliphatic, aromatic and indole according to their chemical structure (Holst and Williamson 2004). Even though intact GLS are relatively biologically inactive, their hydrolysis products such as ITCs, nitriles, thiocyanates, epithionitriles and oxazolidinethiones have numerous therapeutic properties (Fahey et al. 2001; Kim and Lee 2009).

2.4.2 Quorum sensing inhibition by phytochemicals: a new attractive approach against bacterial pathogens and their biofilms

The treatment of biofilm-related infections remains a significant medical challenge. The eradication of biofilms with single target antimicrobials is difficult to accomplish, and synergism between distinct bacterial targets is required for the treatment of biofilm infections (Simões 2011). Currently, several researchers were able to identify improved strategies for

biofilm control (Simões et al. 2009; Landini et al. 2010; Simões 2011). In fact, as previously reported by Simões et al. (2009), the use of phytochemicals may act as a natural antimicrobial strategy with significant impact not only against planktonic bacteria but also on bacterial biofilm formation.

Recent findings indicate that some natural phenolic compounds commonly found in ginger rhizome (*Zingiber officinale Roscoe*), such as [6]-gingerol, [6]-shogaol and [6]-azashogaol, a new derivative of [6]-shogaol, possess QSI activity against *C. violaceum* and *P. aeruginosa* (Kumar et al. 2014).

Crude extracts of garlic have been shown to inhibit the expression of a large number of QS-controlled genes. Jakobsen et al. (2012b) demonstrated the ability of the primary QS inhibitor present in garlic, ajoene, to attenuate in a concentration-dependent manner a few but central QS-controlled virulence factors. This compound also demonstrated a synergistic, antimicrobial effect with tobramycin on biofilm killing and a cease in lytic necrosis of polymorphonuclear leukocytes. Moreover, a significant clearing of infecting *P. aeruginosa* was detected in ajoene-treated mice.

In a study carried out by Jakobsen et al. (2012a), the ITC iberin from horseradish was identified as a potent QS inhibitor. Experiments revealed that iberin specifically and effectively targets two of the major QS networks in *P. aeruginosa*, the *LasI/R* and the *RhlI/R* systems, and downregulates QS-controlled rhamnolipid production in *P. aeruginosa* wild-type batch cultures.

Glycosylflavonoids were also shown to possess QSI potential. Brango-Vanegas et al. (2014) showed the capacity of the compounds chlorogenic acid, isoorientin, orientin, isovitexin, vitexin, and rutin to act as QS inhibitors, using the biosensors *Escherichia coli* pSB403 and *C. violaceum* ATCC 31532.

Packiavathy et al. (2014) evaluated the antibiofilm and anti-QS activities of curcumin from *Curcuma longa* in the prevention of urinary tract infection-causing pathogens *E. coli, P. aeruginosa* PAO1, *Proteus mirabilis* and *Serratia marcescens*. Curcumin was shown to inhibit the biofilm formation of all the studied uropathogens, most likely by interfering with their QS systems. The treatment with curcumin also attenuated the QS-dependent factors, such as alginate production, exopolysaccharide production, and swimming and swarming motility of uropathogens. Furthermore, it was reported that curcumin enhanced the susceptibility of uropathogens to conventional antibiotics.

Gilabert et al. (2015) evaluated the ability of ten *Lepidozia chordulifera* terpenoids to control bacterial growth, biofilm formation, QS process, and elastase activity of *P. aeruginosa* as well as bacterial growth and biofilm formation of *S. aureus.* The aromadendrane-type

sesquiterpenoid viridiflorol was found to be the most potent biofilm formation inhibitor, producing 60% inhibition in *P. aeruginosa* and 40% in *S. aureus*. Betulinic and ursolic acids were able to reduce 92% and 96% in the elastase activity of *P. aeruginosa*, respectively. From all the analyzed triterpenoids, those carrying a dammarane skeleton were the most potent inhibitors of the *P. aeruginosa* biofilm formation and were active against both *P. aeruginosa* and *S. aureus.*

In a study carried out by Castillo-Juarez et al. (2013), experiments were performed to test the potential of an anacardic acids mixture (AAM) on the inhibition of QS-controlled virulence factors expressed on the pathogenic bacteria *P. aeruginosa*. AAM inhibited 86% and 91% of pyoacyanin and rhamnolipid production, respectively, in a dose/response manner and a 75% decrease in elastase activity, without affecting cell growth.

2.4.3 Structural modification: an approach to enhance bioactivity

After a significant innovation gap towards the discovery of new anti-infective compounds due to the application of wrong discovery strategies and deprioritization of many pharmaceutical companies, a renewed interest in the chemical diversity of natural products is drawing researchers' attention. In this sense, several phytochemicals have been screened revealing unique properties, when compared with the synthetic ones, being a new hope to overcome the bacterial resistance problem. As illustrated by some studies, phytochemicals are an ideal source of scaffolds for novel drugs (Simões et al. 2009). The usefulness of these molecules is also corroborated by the fact that many of the current pharmaceutical products in clinical use have plant origins (Saleem et al. 2010). However, despite the high number of compounds with therapeutic properties found in plants, many of them may not be usable due to inappropriate characteristics to be considered as drugs. For instance, the concentrations of phytochemical required are too high to have clinical interest, they do not display selective toxicity to bacteria or lack the desired pharmacokinetic properties (Simões et al. 2009). Thus, one possible strategy to translate phytochemicals into more functional drugs is through the enhancement of their potency, selectivity and drug-like properties. This can be achieved by structure activity relationship (SAR) studies. In this way, it is possible to identify the structural variables that improve the efficacy of the molecule. For instance, parameters such as potency and drug-like properties are highly dependent of the physical/chemical properties of phytochemicals which in turn is related with the type/number of functional groups and their location in the molecule (Borges et al. 2015).

In conclusion, phytochemicals can constitute new scaffolds for drug discovery and development programs that can be tailored for fine-tuning their drug-like properties and so replenish the antibiotic pipeline (Newman and Cragg 2009).

CHAPTER 3

Quorum sensing inhibition activity of selected phytochemicals and derivatives against *Chromobacterium violaceum*

3.1 Introduction

The discovery and elucidation of the mechanisms underlying cell-to-cell communication in bacteria has offered promising novel targets for antivirulence therapies aimed at disarming rather than at eradicating bacterial pathogens during the course of colonization and infection. In fact, many Gram-positive and Gram-negative bacteria use QS to coordinate behaviors and regulate a wide array of physiological activities including swarming, symbiosis, virulence, competence, production of extracellular enzymes, antibiotic synthesis and biofilm formation (Busetti et al. 2015). Because a large number of systems affecting pathogenicity are controlled by QS, their interruption represents an emerging and promising strategy that renders pathogens more susceptible to antibiotics and hinders their adaptation to host immune response. Besides, the discontinuation of this communication attenuates bacterial virulence by limiting the emergence of pathogenic traits (Castillo-Juarez et al. 2013; Busetti et al. 2015).

C. violaceum is a Gram-negative, facultative anaerobic, oxidase-positive bacillus (Hagiya et al. 2014). It is a natural inhabitant ubiquitously distributed in soil and water, and its growth is significantly affected by temperature (Yang 2011). Infection due to *C. violaceum* is rare and sporadic with fewer than 200 cases reported worldwide so far, most of them in geographically confined areas such as tropical/subtropical regions (Hagiya et al. 2014).

Despite its low pathogenicity, *C. violaceum* wild-type and corresponding bioreporter mutants are used as model organisms in diverse studies related to the bacterial communication systems due to its ability to produce a water-insoluble purple pigment named violacein (Hagiya et al. 2014). The production of this pigment is regulated by an AHL-based QS system which comprises a transcriptional regulator (*CviR*) and its cognate signals (N-hexanoyl-L-homoserine lactone (C6-AHL) and N-butanoyl-L-homoserine lactone (C4-AHL) synthetized by the AHL synthase (*CviI*) (LaSarre and Federle 2013). During bacterial growth phase, the AIs accumulate in the extracellular environment and when a threshold concentration is achieved, they bind to *CviR* and this complex triggers violacein production (Blosser and Gray 2000; Borges et al. 2014a). Thus, any change in the pigment production by *C. violaceum* under the influence of

certain products can be visually observed and quantified spectrophotometrically (Chaudhari et al. 2014).

Several studies have been reported the ability of plant-derived products to interfere with the QS system of *C. violaceum* (Castillo-Juarez et al. 2013; Borges et al. 2014a; Chaudhari et al. 2014), demonstrating the reliability of these assays for initial screenings. Hence, the potential of thirteen selected phytochemicals and five derivatives (Table A1 in appendix) to inhibit the aforementioned *C. violaceum* QS system was exploited. The QSI activity of the tested compounds was evaluated using a disc diffusion assay based on violacein inhibition. In addition, extraction and quantification of this pigment were carried out. The mechanisms of QSI based on the modulation of AHLs activity and synthesis by the phytochemicals were also investigated.

3.2 Materials and methods

3.2.1 Microorganisms and culture conditions

C. violaceum CV12472 produces and responds to the AIs C6-AHL and C4-AHL, and was used to determine QSI activity. *C. violaceum* CV31532 (an overproducer of AI C6-HSL) and CV026 (a miniTn-5 mutant of the wild strain CV31532, which is not capable of producing its own AHL molecules, but responds to exogenous active signal molecules C4-AHL and C6-AHL) were used to evaluate the modulation of AHL activity and synthesis (McClean et al. 1997; Choo et al. 2006). All strains were kindly provided by Professor Robert McLean (Texas University, USA). The bacteria were routinely cultured aerobically in Luria–Bertani broth (LBB; Liofilchen, Italy) at 30 °C with agitation (150 rpm) in a shaking incubator (KS 130 basic, IKA, Germany), prior to experiments. For incubation periods during the experiments, the aforementioned conditions of temperature and agitation were also implemented. Luria-Bertani (LB; Liofilchen, Italy) agar (LBA; Merck, Germany) was used to test the activity of selected phytochemicals and derivatives on QSI and on modulation of AHL activity/synthesis.

3.2.2 Phytochemicals and derivatives

The selected phytochemicals for the determination of QSI activity were: CAPE, CAPE derivatives (CABn, NBn, CNBn, NCAPE and CNCAPE), RA, SA, SYR, BE, PIPE, PIAC, MYR, OCI, CITA, CITO, CARV, and FUR. RA and SYR were obtained from Sigma-Aldrich (United Kingdom), SA from Alfa Aesar (Germany), BE from Cayman Chemical Company (USA), MYR from Acros Organics (France), OCI and CARV from Sigma-Aldrich (USA), CITA from Acros Organics (Spain), and CITO from Acros Organics (USA). The remaining

compounds (FUR, CAPE and its derivatives, PIPE, and PIAC) were gently provided by Prof. Dr. Fernanda Borges (Faculty of Sciences of the University of Porto, Portugal). Stock solutions of all tested compounds were prepared with dimethyl sulfoxide (DMSO; Fisher Scientific, United Kingdom) under sterile conditions, and serial dilutions were prepared when needed. The percentage of solvent always stood in 10% (v/v) of the final volume of cell suspension.

3.2.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined by the microdilution method according to Borges et al. (2012). Briefly, bacteria were grown overnight in LBB at 30 °C and 150 rpm and then an inoculum was taken and adjusted to an absorbance value of 0.1 ± 0.02 (A_{620}). Afterwards, 96-well polystyrene (PS) microtiter plates were filled with cells (180 µL) and phytochemicals (20 µL) in a range of different concentrations (6.25 to 1000 µg/mL), followed by their incubation for 24h at 30 °C and 150 rpm. Cell suspension with DMSO and cell suspension without phytochemical were used as negative controls. Absorbance measurements of the microtiter plates' content were performed before (t = 0 h) and after (t = 24 h) the incubation period using a microplate reader (Synergy HT, Biotek, United States of America). The MIC was recorded as the lowest concentration of tested compounds which showed no difference between the absorbance values measured at both distinct times (cell growth inhibition). After MIC determination, a volume of 10 µL of each well corresponding to the phytochemical and/or derivative concentrations equal and above the MIC was taken and plated out on LBA. Plates were incubated at 30 °C for 24 h and the growth was visually inspected. The MBC was recorded as the lowest concentration of phytochemical/derivative in which total growth inhibition was observed (Ferreira et al. 2011). All tests were performed in duplicate with six repeats.

3.2.4 Bioassay for detection of quorum sensing inhibition

A qualitative standard disc diffusion assay (Bauer et al. 1966) based on the violet pigment inhibition was performed with the bioindicator strain *C. violaceum* CV12472 to detect the QSI activity of the selected phytochemicals. Concentrations lower than, equal to, and higher than the MIC were tested. Briefly, LBA plates were inoculated with 100 µL of an overnight culture of *C. violaceum* CV12472 adjusted to an absorbance value of 0.1 ± 0.02 (A_{620}) (1.4×10^8 colony forming units (CFU)/mL). Afterwards, sterile paper discs (6 mm in diameter) (Oxoid, Spain) were placed over the plates which were loaded with 10 µL of different concentrations of each

compound. DMSO and LBB were used as negative controls. After incubation for 24 h at 30 °C, the inhibition of the pigment production around the disk (a ring of colorless but viable cells) was checked. Antimicrobial activity was indicated by the lack of microbial growth. Bacterial growth inhibition was measured as diameter 1 (d_1) in mm while both bacterial growth and pigment inhibitions were measured as total diameter 2 (d_2) in mm. Thus, QSI, assessed by pigment inhibition, was determined by subtracting the diameter of bacterial growth inhibition (d_1) from the total diameter (d_2) (QSI = d_2 - d_1), according to Zahin et al. (2010).

3.2.5 Violacein extraction and quantification

After the initial screening, using the qualitative agar disc diffusion method, QSI caused by phytochemicals and derivatives was also quantified in a broth assay, according to Choo et al. (2006) (this assay was only performed for compounds with positive QSI activity). For this test, only the concentrations of phytochemicals and derivatives which demonstrated the ability for QSI in the disc diffusion assay were used. *C. violaceum* CV12472 cells (A_{620} = 0.1) supplemented with different concentrations of phytochemicals and derivatives were incubated for 24 h (30 °C and 150 rpm). Then, violacein extraction was carried out. Briefly, 1 mL of culture from each flask was centrifuged (Centrifuge 5424, Eppendorf, Germany) at 14549 g for 10 minutes to precipitate the insoluble violacein and bacterial cells. The culture supernatant was discarded and 1 mL of DMSO was added to the pellet. The solution was vortexed vigorously for 30 seconds to completely solubilize violacein, and centrifuged at 14549 g for 10 minutes to remove the cells. Two hundred microliters of the violacein-containing supernatants were added to 96-well polystyrene microtiter plates and the absorbance was read with a microplate reader at a wavelength of 585 nm. The results were expressed as percentages of violacein reduction and cell growth inhibition. This assay was performed with four repeats.

3.2.6 Modulation of AHL activity and synthesis

The agar diffusion double ring assay was used to evaluate the effect of the phytochemicals and derivatives on the modulation of both AHL activity and synthesis, as described previously (McClean et al. 1997). For this test, only the concentrations of compounds which demonstrated the ability for QSI in the disc diffusion assay were used. After overnight growth, *C. violaceum* CV31532 and *C. violaceum* CV026 cultures were adjusted to an absorbance value of 0.1 ± 0.02 (A_{620}) and subsequently assayed for violacein production. Sterile paper discs were placed at the center of the LBA plates and were loaded with 10 μL of different

concentration of compounds. Controls with DMSO and LBB were performed. To test for AHL activity, the biosensor *C. violaceum* CV026 was streaked in a circle on the LBA plates in close proximity to the disc and the overproducer *C. violaceum* CV31532 was streaked on the outside (4-5 mm distance between the two biosensors). To assess the inhibition of AHL synthesis, the location of the AHLs overproducer and biosensor strains was inverted. The plates were incubated for 24 h at 30 °C and the production of violacein was visually inspected. The tests were performed in duplicate with two repeats.

3.2.7 Statistical analysis

The results were analyzed using paired samples t-test from the Microsoft Excel 2010. Statistical calculations were based on a confidence level of ≥ 95% ($p < 0.05$ was considered statistically significant).

3.3 Results and discussion

3.3.1 Antimicrobial activity of selected phytochemicals

In order to know the antimicrobial activity of the compounds selected for this study, as well as to choose the concentrations that will be used in QSI assays, the MIC and MBC were determined. The MIC and MBC values obtained for the tested phytochemicals and derivatives against the bioindicator strain *C. violaceum* CV12472 are presented in Table 1. The MIC and MBC of the selected compounds ranged from 12.5 to > 1000 µg/mL and 25 to > 1000 µg/mL, respectively. No inhibitory and bacteridical effects were observed with all phenolics tested (RA, SA, SYR) (MIC/MBC > 1000 µg/mL), except with CAPE (MIC = 150 µg/mL and MBC = 180 µg/mL). Regarding CAPE derivatives, NBn had the lowest MIC (90 µg/mL) and CNCAPE the highest one (600 µg/mL). For the remaining CAPE derivatives, CABn, CNBn, and NCAPE, the MIC values were 250 µg/mL, 110 µg/mL, and 180 µg/mL. In terms of bactericidal effects, the MBC found for CAPE derivatives, in the increasing order of activity were CNBn < CABn < NCAPE < NBn < CNCAPE. Both alkaloids tested, BE and PIPE, had antimicrobial activity with distinct MIC values, the first one showed a MIC of 800 µg/mL and the second one a MIC 4 times lower (200 µg/mL). Conversely, these compounds showed no bactericidal activity (MBC > 1000 µg/mL). PIAC, an analogue based on PIPE scaffold, displayed MIC/MBC > 1000 µg/mL. Regarding the terpenoids class, the EO components, OCI and CITO exhibited the lowest MICs (12.5 and 25 µg/mL, respectively), followed by CITA/MYR (100 µg/mL) and CARV (200 µg/mL). FUR, a nitrovinylfuran, displayed a MIC and MBC of 400 µg/mL. In

general, OCI and CITO were the most effective compounds assayed with respect to inhibitory and bactericidal activity, respectively.

Table 1. MIC and MBC values of selected phytochemicals and derivatives against *C. violaceum* CV12472.

Phytochemical		***C. violaceum* CV12472**	
		MIC (µg/mL)	**MBC (µg/mL)**
CAPE		150	180
CAPE derivatives	CABn	250	250
	NBn	90	380
	CNBn	110	220
	NCAPE	180	360
	CNCAPE	600	740
RA		NA	NA
SA		NA	NA
SYR		NA	NA
BE		800	NA
PIPE		200	NA
PIAC		NA	NA
MYR		100	NA
OCI		12.5	NA
CITA		100	200
CITO		25	25
CARV		200	400
FUR		400	400

(NA) – No activity, the MIC/MBC is higher than the maximum concentration tested (>1,000 µg/mL).

3.3.2 QSI of selected phytochemicals and derivatives

A disc diffusion assay was performed for QSI screening using the biosensor *C. violaceum* CV12472. In this way, a range of concentrations (from sub-MICs to >MICs) were tested to ascertain whether the halos produced around the disks were due to growth inhibition of cells and/or QSI. Hence, loss of purple pigment by *C. violaceum* CV12472 is indicative of QSI by the selected phytochemicals and derivatives. The results are displayed in Table 2.

Among the eighteen compounds tested, only five displayed QSI activity, including CABn, PIAC, CITO, CARV and FUR (Figure 3).

The CAPE derivative, CABn, exhibited QSI halos of 3 mm (25 µg/mL and 50 µg/mL), and 7 mm (100 µg/mL) and antimicrobial halos of 4 mm for the same concentrations. The analysis of the structure of CABn and its QSI shows a higher potential of the benzyl group compared to the radical CH_2CH_2Ph present in CAPE. Besides, it is possible to verify that the

presence of both nitro and cyano groups on benzene ring did not enhance the QSI potential of CAPE derivatives.

PIAC, a synthetic compound obtained by alkaline hydrolysis of PIPE, caused partial pigment inhibition at 800 and 1000 µg/mL with QSI halos of 5 and 7 mm, respectively, and presented very little antimicrobial activity (maximum of 2 mm). Hence, the substitution of the piperidine group present in PIPE for a hydroxyl group in PIAC seems to enhance QSI activity.

CITO and CARV, both members of the terpenoids group, demonstrated potential to interfere with the QS system of *C. violaceum* but also to inhibit its growth. Similar results were observed in other works, with extracts and EOs from medicinal plants. (Adonizio et al. 2006; Khan et al. 2009; Koh and Tham 2011) CITO presented QSI halos of 4 mm (800 and 1000 µg/mL) and CARV displayed QSI halos of 3 mm (400 µg/mL), 7 mm (800 µg/mL), and 8 mm (1000 µg/mL). In addition, antimicrobial activity was also detected with the aforementioned phytochemicals. CITO presented antimicrobial halos of 6 mm (800 µg/mL) and 13 mm (1000 µg/mL) and CARV displayed halos of 5 mm (400 µg/mL), 9 mm (800 µg/mL) and 11 mm (1000 µg/mL). When compared to CITA, the chemical structure of CITO differs solely on its hydroxyl group placed instead of a formyl group. In addition, CARV possesses a cyclic structure, a unique feature within the tested terpenoids, with also a hydroxyl group. Thus, it is possible to infer that terpenoids with hydroxyl groups and cyclic structures have a higher potential for QSI. Indeed, several reports suggest that the presence and location of functional groups in the molecule of EOs components, can affect their bioactivity (Nazzaro et al. 2013).

The biggest QSI halos within all the tested phytochemicals and derivatives belong to FUR. Partial visualization of pigment color was reported even at the lowest concentration tested (halo of 7 mm), reaching a maximum halo value of 15 mm at 25 µg/mL. This value was remained more or less constant until a concentration of 100 µg/mL, ending up in 7 mm for a concentration of 1000 µg/mL. Antimicrobial activity was also detected for this compound, with halos ranging from 6 to 51 mm.

Although QSI activity has been observed with all the aforementioned compounds, this activity need to be further characterized with respect to the genes that are affected by the phytochemicals.

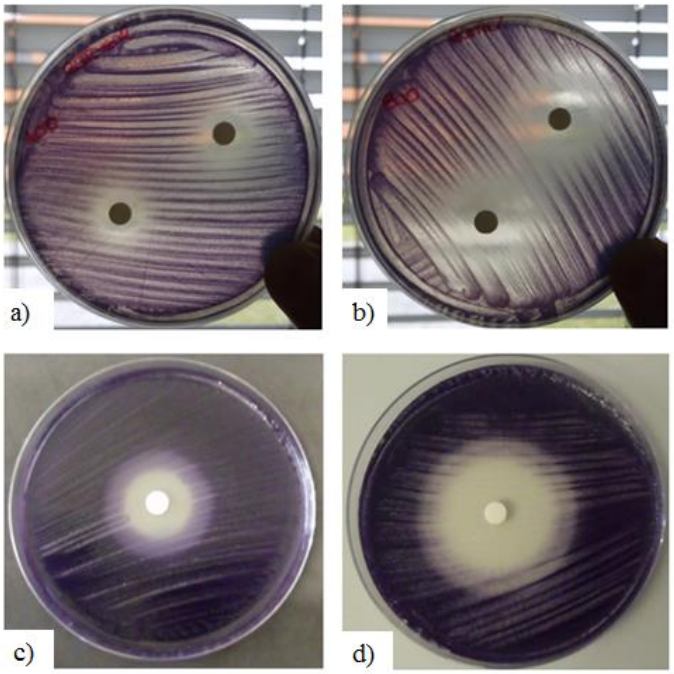


Figure 3. QSI and antimicrobial halos caused by CARV ((a) 400 µg/mL and (b) 800 µg/mL) and FUR ((c) 50 µg/mL and (d) 100 µg/mL) against *C. violaceum* CV12472.

Regarding all the remaining compounds, no QSI was detected at the tested concentrations. CAPE and four of its derivatives (NBn, CNBn, NCAPE, and CNCAPE) did not show any pigment inhibition and very little antimicrobial activity was observed (halos ranging from 2 to 4 mm). Within the phenolic group (RA, SA, SYR) no pigment inhibition was observed at the tested concentrations. However, growth inhibition halos between 3 and 6 mm, 2 and 7 mm, and 6 and 7 mm were detected for RA, SA and SYR, respectively. Indeed, Borges et al. (2014a) found that ferulic and gallic acids, structurally similar to sinapic and syringic acids, respectively, did not interfere with the QS system of *C. violaceum* but inhibited its growth. Besides, the authors suggest that the lack of QSI detection of tested phenolics can be due to the limitation of the QSI assay used. In the same way, in the present study, the compounds tested can affect QS systems in a different way from that of *C. violaceum*. Effectively, some studies showed positive interference of various phenolics with the QS systems of *E. coli*, *Pseudomonas putida* and *P. aeruginosa* (Huber et al. 2003; Singh et al. 2009; Yang et al. 2009). BE and PIPE showed no pigment inhibition and very little antimicrobial activity (growth inhibition halos up to 2 mm) was detected. The hypothesis proposed for the compounds mentioned above can be also the explanation of the lack of activity observed for these alkaloids. Indeed, Cech et al. (2012) found that *Hydrastis canadensis* leaf extract, which contains alkaloids such as BE, possesses QSI activity against methicillin-resistant *S. aureus*. The terpenoids MYR, OCI and CITA revealed no decrease on pigment production. Yet, CITA exhibited antimicrobial activity, with halos ranging from 8 to 21 mm.

Table 2. Antimicrobial (AM) and QSI activities against *C. violaceum* CV12472.

Phytochemical		Concentration (µg/mL)																			
		LB	DMSO	0.5	1	2.5	5	6.25	10	12.5	20	25	35	45	50	75	100	200	400	800	1000
CAPE	PP	+	+	N/A	+	+	+	N/A	+	N/A	N/A	+	N/A	+	N/A	N/A	+	+	+	+	+
	AM	0	0	N/A	2	2	2	N/A	2	N/A	N/A	2	N/A	4	N/A	N/A	3	3	3	3	3
	QSI	0	0	N/A	0	0	0	N/A	0	N/A	N/A	0	N/A	0	N/A	N/A	0	0	0	0	0
CABn	PP	+	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+/-	N/A	N/A	+/-	N/A	+/-	N/A	N/A	N/A	N/A
	AM	0	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4	N/A	N/A	4	N/A	4	N/A	N/A	N/A	N/A
	QSI	0	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	N/A	N/A	3	N/A	7	N/A	N/A	N/A	N/A
NBn	PP	+	+	+	+	+	+	N/A	+	N/A	+	N/A	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	AM	0	0	0	2	2	2	N/A	2	N/A	2	N/A	3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	QSI	0	0	0	0	0	0	N/A	0	N/A	0	N/A	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CNBn	PP	+	+	+	+	+	+	N/A	+	N/A	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	AM	0	0	2	2	2	0	N/A	2	N/A	3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	QSI	0	0	0	0	0	0	N/A	0	N/A	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NCAPE	PP	+	+	+	+	+	+	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	N/A	N/A	N/A	N/A
	AM	0	0	3	2	2	2	N/A	2	N/A	2	N/A	3	N/A	N/A	2	N/A	N/A	N/A	N/A	N/A
	QSI	0	0	0	0	0	0	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	N/A	N/A	N/A	N/A
CNCAPE	PP	+	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+	N/A	N/A	+	N/A	N/A	N/A	N/A	N/A
	AM	0	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	N/A	N/A	2	N/A	N/A	N/A	N/A	N/A
	QSI	0	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	N/A	N/A	0	N/A	N/A	N/A	N/A	N/A
RA	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	3	N/A	4	4	4	6	6
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0
SA	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	2	2	2	3
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0
SYR	PP	0	0	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	6	7
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0

BE	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	N/A	N/A
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	2	N/A	2	N/A	N/A	2	N/A	2	2	0	N/A	N/A
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	N/A	N/A
PIPE	PP	+	+	N/A	N/A	N/A	N/A	N/A	+	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	N/A	2	2	N/A	2	N/A	N/A	2	N/A	2	2	2	2	2
	QSI	0	0	N/A	N/A	N/A	N/A	N/A	0	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0
PIAC	PP	+	+	+	+	+	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	N/A	+	+/-	+/-
	AM	0	0	2	2	2	2	N/A	2	N/A	2	N/A	N/A	2	N/A	2	2	N/A	2	2	2
	QSI	0	0	0	0	0	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	N/A	0	5	7
MYR	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	2	N/A	2	2	3	4	4
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0
OCI	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	2	N/A	2	N/A	2	N/A	N/A	2	N/A	3	3	2	2	3
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0
CITA	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+	+
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	5	N/A	8	13	16	20	21
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	0	0
CITO	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+	+/-	+/-
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	3	N/A	2	N/A	N/A	6	N/A	3	3	5	6	13
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	0	4	4
CARV	PP	+	+	N/A	N/A	N/A	N/A	+	N/A	+	N/A	+	N/A	N/A	+	N/A	+	+	+/-	+/-	+/-
	AM	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	3	5	5	9	11
	QSI	0	0	N/A	N/A	N/A	N/A	0	N/A	0	N/A	0	N/A	N/A	0	N/A	0	0	3	7	8
FUR	PP	N/A	N/A	N/A	N/A	N/A	N/A	+/-	N/A	+/-	N/A	+/-	N/A	N/A	+/-	N/A	+/-	+/-	+/-	+/-	+/-
	AM	N/A	N/A	N/A	N/A	N/A	N/A	0	N/A	0	N/A	6	N/A	N/A	14	N/A	22	38	41	51	50
	QSI	N/A	N/A	N/A	N/A	N/A	N/A	7	N/A	10	N/A	15	N/A	N/A	13	N/A	14	11	11	7	7

Note: The diameter of both QSI and AM halos are presented in mm. (N/A) – Not applicable; (PP) – Pigment production; (+) – visualization of pigment color; (+/-) – partial visualization of pigment color.

3.3.3 Quantification of the amount of violacein inhibited by the selected phytochemicals

Although the disc diffusion method is effective for the detection of QS inhibitors, it is not possible to quantify the exact amount of violacein inhibition. With the aim of reinforcing the results obtained in the qualitative assay related to pigment inhibition, violacein was extracted and quantified. Figure 4 shows the results of violacein and cell growth inhibitions by CITO and CARV. Due to the low efficacy of the synthetic procedure of CABn (lack of available derivative) it was not possible to quantify the amount of violacein inhibition for this compound. Besides, although PIAC and FUR have been synthesized, solubility problems precluded their use, and thus violacein quantification was not carried out.

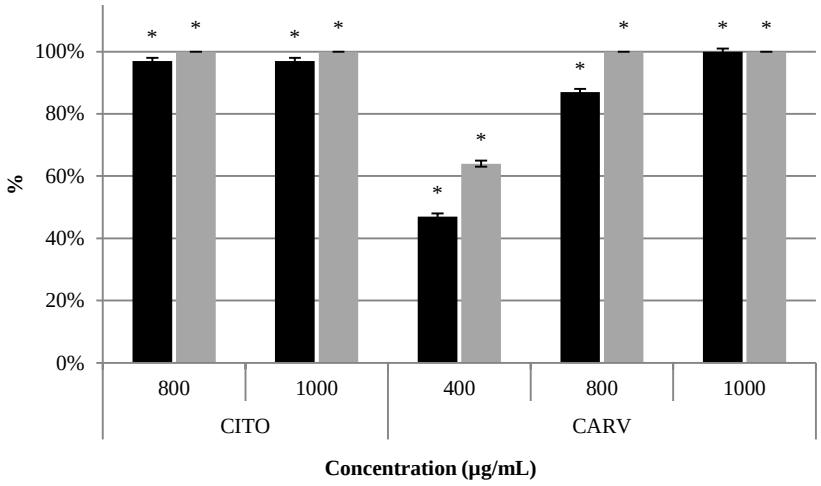


Figure 4. Influence of CITO and CARV in violacein (black) and cell growth (grey) inhibitions. The results are presented as percentages of violacein and cell growth inhibitions. Mean values ± SD for four repeats are illustrated. Bars with * are statistically different from the control ($p < 0.05$).

Violacein production was inhibited by more than 90% with CITO for all the tested concentrations ($p < 0.05$). The same behavior was verified with CARV. For a concentration of 400 µg/mL, the percentage of violacein inhibition was around 45%. At 800 µg/mL, violacein inhibition reached up to almost 90% and at 1000 µg/mL it exhibited a value of 100%. However, the percentages of cell growth inhibition were always higher compared to percentages of violacein inhibition. Hence, it seems that in this assay violacein inhibition was due to inhibition of bacterial growth/bacterial death rather than inhibiton of violacein synthesis. These results are corroborated by those obtained in the disc diffusion assay, where CITO and CARV produced halos of growth inhibition in addition to QSI.

3.3.4 QSI based on modulation of AHL activity and synthesis

The common mechanisms of QS interference include: inhibition of signal biosynthesis or inhibition of activity of AHL-producing enzymes; enzymatic signal degradation; and inhibition of reception signal molecules (Zhang and Dong 2004; Rasmussen and Givskov 2006; Khan et al. 2009). In this study, the effects of CITO and CARV on modulation of AHL activity (via LuxR-type receptor) and synthesis (via LuxI-type AHL synthases) were investigated. For this assay, the concentrations with a positive effect on violacein inhibition in the disc diffusion assay were selected. AHL activity was assessed by the decrease in violacein pigment production in *C. violaceum* CV026 AHL biosensor, due to low levels of QS related to AHL detection. AHL synthesis was also evaluated by pigment inhibition of *C. violaceum* CV026, due to the decrease or absence of AHL production by the overproducer *C. violaceum* CV31532 in the presence of the phytochemicals.

Table 3 shows the results of modulation of AHL activity and synthesis by CITO and CARV.

Table 3. Modulation of AHL activity and synthesis by CITO and CARV at different concentrations.

Phytochemical	Concentration (µg/mL)	*C. violaceum* CV026	
		Modulation of AHL activity	Modulation of AHL synthesis
LB control	N/A	++++	++++
DMSO control	N/A	++++	++++
CITO	800	+++	+++
	1000	+++	++
CARV	400	+++	+++
	800	+++	++
	1000	+++	++

(N/A) – not applicable; (++++) – very intense pigment color; (+++) – intense pigment color; (++) – little intense pigment color; (-) – absence of pigment color.

The results demonstrated that CITO and CARV had the capacity to interfere with the synthesis of the AHLs produced by *C. violaceum* CV31532. For CITO, the highest inhibition of pigment color was observed at 1000 µg/mL, whereas for CARV the highest violacein inhibition was more or less constant throughout every tested concentration except at 800 µg/mL. Although CITO and CARV have interfered with the violacein intensity on the modulation of AHL activity, its reduction was not enough to infer about the ability of these phytochemicals to affect AHL activity.

QSI by CARV and CITO can be achieved in several ways: they could affect the QS signaling cascade of *C. violaceum* CV31532 strain, binding directly to the LuxR-type receptor by competing with the AHL molecules and/or by preventing the binding of the AHL molecules to these receptors (Hentzer and Givskov 2003; Rasmussen et al. 2005a; Rasmussen et al. 2005b).

Since CITO and CARV were effective at inhibiting QS mediated by two different AHL producers (*C. violaceum* CV12472 and CV31532), it may be assumed that these compounds are able to inhibit multiple bacterial QS system homologues of LuxI/LuxR systems which are mediated by AHL molecules.

3.4 Conclusions

In the present study, CABn, PIAC, CITO, CARV and FUR were found to be the most promising compounds in targeting the QS system of *C. violaceum*. QSI activity was observed for all the aforementioned phytochemicals/derivatives. Additionally, the EOs components CITO and CARV demonstrated their ability to interfere with the synthesis of the AHLs.

So far, no documented information about the potential QSI activity of these compounds is known. Even though these phytochemicals/derivatives may have potential to be developed as new QS inhibitors to treat emergent infections, including those biofilm related, additional studies are demanded in order to test, for instance, their toxicity. Interestingly, FUR is commercially sold as a broad-spectrum antibiotic against bacteria and fungi. Hence, the QSI potential of this drug suggests that a new mode of action is about to born.

CHAPTER 4

Aspects underlying *Pseudomonas aeruginosa* pathogenicity

4.1 Introduction

P. aeruginosa is a Gram-negative opportunistic pathogenic bacillus, associated with biofilm-related nosocomial infections such as ventilator-associated pneumonia and chronical lung infection in cystic fibrosis sufferers. Together, these infections constitute the major cause of patient morbidity and mortality (Busetti et al. 2015). The relationships among QS, virulence regulation, and biofilm formation have most extensively been studied in *P. aeruginosa*. Therefore, it is not surprising that most of the research on QS inhibition has been centered on this bacterium as a model system (Imperi et al. 2013).

The high level of *P. aeruginosa* pathogenicity is mainly due to the expression of QS-controlled virulence factors and the formation of recalcitrant biofilms, which endow the bacterial cells with protection from the host defense system and resistance to many antimicrobial agents (Davies et al. 1998; Cathcart et al. 2009).

P. aeruginosa utilizes two different QS circuits to regulate the production of virulence factors and promote biofilm maturation (Busetti et al. 2015). These are termed the *LasI/R* and *Rh1I/R* systems (Popat et al. 2008). Each of these systems comprises a transcriptional regulator (*LasR* and *Rh1R*, respectively) and its cognate AHL signal (*N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL), respectively), synthesized by the AHL synthase (*LasI* or *RhlI*, respectively) (Le Berre et al. 2008; de Kievit 2009). More specifically, *LasI* directs the synthesis of 3-oxo-C12-HSL which activates the transcriptional regulator *LasR* and in turn *LasR*/3-oxo-C12-HSL complex induces the production of virulence factors including elastase, the *LasA* protease, alkaline protease and exotoxin A. *Rh1I* directs the synthesis of C4-HSL that together with the transcriptional regulator Rh1R regulates the production of virulence factors namely the production of rhamnolipid, elastase, *LasA* protease, pyocyanin, siderophores, hydrogen cyanide, and the cytotoxic lectins PA-I and PA-II. The *LasI/R* and the *RhlI/R* systems are organized in such a way that the *LasI/R* system exerts transcriptional control over both *RhlR* and *RhlI*. In addition to AHL-dependent QS, *P. aeruginosa* also produces over 50 2-alkyl-4(1H)-quinolones. One of these compounds, 2-heptyl-3-hydroxy-4(1H)-quinolone was discovered to act as a diffusible signal molecule and termed PQS (Figure 5). PQS was shown to regulate *P. aeruginosa* virulence gene expression

and to act as an integral component of the QS network since its production is modulated by both the *LasI/R* and the *RhlI/R* systems (Popat et al. 2008).

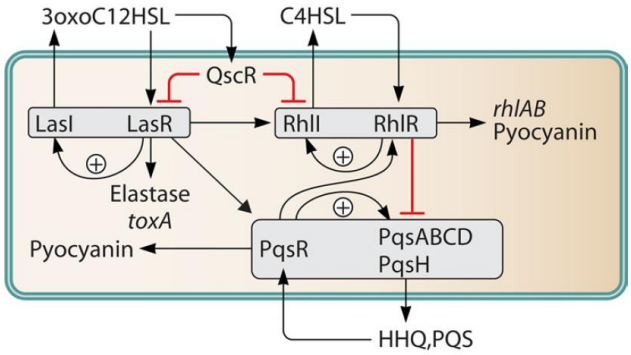


Figure 5. Simplified scheme of *P. aeruginosa* QS network. Arrows imply information flow and T-bars indicate negative regulation, whereas arrows labeled (+) indicate documented positive feedback (LaSarre and Federle 2013).

Therefore, the quest for effective strategies to control *P. aeruginosa* pathogenicity is demanded, particularly with consideration to the increase in antibiotic resistance observed and the difficulty to treat bacterial infections (Stewart and Costerton 2001; Hoffman et al. 2005).

As a therapeutic candidate, much attention has been focused in targeting the QS regulatory mechanism of this bacterium due to the critical role of QS in virulence expression and biofilm formation (Smith and Iglewski 2003), as previously mentioned. In fact, previous studies have shown that it is possible to decrease the antibiotic tolerance and attenuate virulence of *P. aeruginosa* biofilms by antagonistic perturbation of the QS signaling system (Jakobsen et al. 2012a; Jakobsen et al. 2012b). However, despite the massive efforts made to date in the field of anti-QS research, clinical applications remain far away.

In this chapter, the potential of selected phytochemicals/derivatives (table A1 in appendix) to interfere with the QS system (*LasI/R*) of *P. aeruginosa* was exploited. The effects of QS inhibitors on biofilm development and aspects related with its formation (initial adhesion) and its structure were also studied. Moreover, it was tested the susceptibility of *P. aeruginosa* biofilms (grown in the presence of QS inhibitors) to the antibiotic ciprofloxacin. Finally, the influence of QS inhibitors on production of virulence factors controlled by QS was ascertained.

4.2 Materials and methods

4.2.1 Microorganisms and culture conditions

P. aeruginosa PA14 wild-type and the reporter strain *P. aeruginosa* PA14-R3 were used to screen QSI activity. The biosensor *P. aeruginosa* PA14-R3 responds to the AI 3-oxo-C12-

HSL that is produced by *P. aeruginosa* PA14 wild-type and emits luminescence (Massai et al. 2011). All strains were kindly provided by Professor Livia Leoni (University Roma Tre, Italy). Both bacteria were routinely cultured aerobically in LBB at 37 °C with agitation at 150 rpm in a shaking incubator, prior to experiments. For incubation periods during the experiments, the aforementioned conditions of temperature and agitation were also implemented.

4.2.2 Phytochemicals and derivatives

The selected phytochemicals/derivatives were: CAPE, CAPE derivatives (CABn, NBn, CNBn, NCAPE and CNCAPE), RA, SA, SYR, BE, PIPE, PIAC, MYR, OCI, CITA, CITO, CARV, PEITC and FUR. PEITC was obtained from Sigma-Aldrich (Portugal). Solutions were prepared with DMSO and percentage of solvent never exceeded 6% (v/v) of the final volume of cell suspension for determination of QSI activity and remained constant (10% (v/v)) for all the other tests.

4.2.3 Determination of MIC and MBC

The MIC was determined by the microdilution method according to Borges et al. (2012), as described in Chapter 3 sub-section 3.2.3. MBC evaluation was performed according to Ferreira et al. (2011) as stated in the sub-section 3.2.3. All tests were performed in duplicate with six repeats.

4.2.4 Bioassay for detection of quorum sensing inhibition

The ability of the selected phytochemicals to interfere with the QS response of opportunistic pathogen *P. aeruginosa* was evaluated. This was achieved by a high-throughput QSI screening system, developed by Massai et al. (2011), based on the co-cultivation of a biosensor strain for 3-oxo-C12-HSL detection, *P. aeruginosa* PA14-R3, and a wild-type *P. aeruginosa* PA14 strain. Bioluminescence emission by *P. aeruginosa* PA14-R3 is induced when detection of the 3-oxo-C12-HSL signal synthesized by the wild-type *P. aeruginosa* PA14 wild-type is accomplished. Therefore, the addition of any molecule with inhibitory activity regarding any process of the 3-oxo-C12-HSL-dependent QS system, including 3-oxo-C12-HSL synthesis, transport, and detection, will reduce the luminescence emitted by *P. aeruginosa* PA14-R3 with respect to a control co-culture without any treatment. *P. aeruginosa* PA14-R3 is a PA14 derivative in which a transcriptional fusion between the LasR-dependent *rsaL* promoter and the

luxCDABE operon was chromosomally integrated at the *attB* neutral site of the chromosome. In addition, the *lasI* gene encoding 3-oxo-C12-HSL synthase was inactivated by transposon insertion in the *P. aeruginosa* PA14-R3 strain (Massai et al. 2011). Briefly, *P. aeruginosa* PA14 wild-type and *P. aeruginosa* PA14-R3 were grown overnight at 37 °C on LBA plates. Afterwards, bacteria were scrapped from the plate surfaces and diluted in LBB to absorbance values (A_{600}) of 0.045 and 0.015 for *P. aeruginosa* PA14-R3 and *P. aeruginosa* PA14 wild-type, respectively (3:1 reporter/wild type ratio). Black 96-well flat, opaque bottomed PS microtiter plates and 96-well flat, clear bottomed PS microtiter plates were aliquot with 180 µL of the co-culture and 20 µL of phytochemical in a range of different concentrations (6.25 to 1000 µg/mL). Cell suspension with DMSO and cell suspension without phytochemical were used as negative controls. The light counts per second (LCPS) and A_{600} were measured after 4 h of growth in a microplate reader. Luminescence values were normalized by dividing LCPS values for A_{600} values. The tests were performed with six repeats.

4.2.5 Prevention of initial cell adhesion and biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanovic et al. (2000). Briefly, 96-wells flat, clear bottomed PS microtiter plates were filled with *P. aeruginosa* PA14 wild-type suspension (180 µL) (A_{600} = 0.04 and A_{600} = 0.004) and phytochemical/derivative (20 µL) in a range of different concentrations (6.25 to 1000 µg/mL). Adhesion assays were carried out as mentioned for biofilms but with different incubation times (2 h instead 24 h). Cell suspension with DMSO and cell suspension without phytochemical/derivative were used as negative controls. The plates were incubated at 37 °C and 150 rpm for 2 h (initial cell adhesion) and 24 h (biofilm formation). After the incubation period, the content of each well was discarded and washed two times with sterile water. The plates were analyzed in terms of biomass formation and metabolic activity by crystal violet (CV; Merck, Germany) and alamar blue (Sigma-Aldrich, Portugal) staining, respectively.

4.2.6 Effect of phytochemicals on biofilm susceptibility to ciprofloxacin

In order to access whether phytochemicals have effects on biofilm susceptibility to antibiotic ciprofloxacin, biofilms were developed in the presence of a range of different phytochemical concentrations (6.25 to 1000 µg/mL) as stated above. Cell suspension with DMSO and cell suspension without phytochemical were used as negative controls. After 24 h of incubation at 37 °C and 150 rpm, 24 h old biofilms were washed twice with saline solution

(NaCl) and exposed to ciprofloxacin (Sigma-Aldrich, China) at concentrations corresponding to 5× and 10× MIC. The plates were incubated for 24 h at 37 °C and 150 rpm. The biofilms were analyzed in terms of biomass and metabolic activity by CV and alamar blue staining, respectively.

4.2.7 Biomass quantification of adhered and biofilm cells by crystal violet staining

The biomass of adhered and biofilm cells was quantified using CV staining, according to Simões et al. (2010). The 2 h-adhered cells/24 h old bacterial biofilms cells were fixed with 250 µL of 96% ethanol (Diprolar, Portugal) for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 µL of 1% (v/v) CV. The excess of stain was gently withdrawn and the dye bound to the adherent cells was resolubilized with 200 µL of 33% (v/v) glacial acetic acid (Chem-Lab, Belgium). Absorbance measurements at 570 nm were performed using a microplate reader.

4.2.8 Metabolic activity quantification of adhered and biofilm cells by alamar blue staining

The modified alamar blue microtiter plate assay was carried out in order to determine the metabolic activity of cells, as reported by Sarker et al. (2007). Alamar blue (7-hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma-Aldrich, Portugal) is a blue non-fluorescent redox dye that can be reduced by the cellular metabolic activity to the highly fluorescent pink resorufin (Sandberg et al. 2009). For the staining procedure, 190 µL of fresh LBB were added to each well of the microtiter plate, followed by 10 µL of alamar blue (0.4 mM). Plates were incubated at room temperature (RT) for 20 minutes in darkness. Fluorescence was measured at excitation and emission wavelengths of 570 nm and 590 nm, respectively, using a microplate reader.

4.2.9 Microscopic analysis of bacterial biofilm

The *in situ* epifluorescence microscopy analysis of bacterial biofilm structure was performed according to Musthafa et al. (2010) with some modifications. Briefly, PS coupons (dimensions of 1 × 1 cm) were placed inside 12-wells flat, clear bottomed PS microtiter plates (prior to use, PS coupons were exposed to UV radiation for 30 min). The wells were then filled with 1.8 mL of *P. aeruginosa* PA14 wild-type suspension (A_{600} = 0.04) and 200 µL of

phytochemical in a range of different concentrations (6.25 to 1000 µg/mL). Cell suspension with DMSO and cell suspension without phytochemical were used as negative controls. The plates were incubated at 37 °C and 150 rpm for 24 h. After incubation time, coupons were removed from the microwells and quickly rinsed three times with 2 mL of 0.85 % NaCl to remove loosely attached cells. The biofilm adhered on each coupon was stained with 20 µL of DAPI (Sigma, Portugal) (at a concentration of 0.5 µg/mL), which stains both viable and non-viable cells, for 10 min in the dark. After incubation, the coupons were mounted with non-fluorescent immersion oil on glass microscope slides. The coupons were then visualized under a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd., Switzerland), with ×100 oil immersion fluorescence objective and a filter with excitation filter 340–380 nm, dichromatic mirror of 400 nm and suppression filter LB 425.

4.2.10 Virulence factors assays

4.2.10.1 Pyocyanin production

Pyocyanin was extracted from culture supernatants and measured as previously described by Essar et al. (1990). Briefly, PA14 wild-type suspension (A_{600} = 0.05) were grown in the presence of different concentrations of selected phytochemical, at 37 °C and 150 rpm for 16 h. After incubation, the cells were harvested by centrifugation (15 min at 12,000 g) and the culture supernatant recovered. Pyocyanin was extracted, by mixing three milliliters of chloroform with 5 mL of culture supernatant, followed by a second extraction into 1 mL of 0.2 M hydrochloric acid (HCl; Fisher Chemical, Belgium) to give a red solution (the chloroform layer was transferred to a fresh tube and mixed with HCl). The A_{520} of the resulting solution was measured to determine the amount of extracted pyocyanin. The amount of pyocyanin, in µg/mL, was calculated by multiplication of the A_{520} values for 17.072.

4.2.10.2 Gelatinase production

The gelatinase activity was carried out as stated by Su et al. (1991). Ten microliters of PA14 wild-type suspension (A_{600} = 0.1) were plated out on plates containing Gelatin Agar (5 g/L peptone (Merck, Darmstadt, Germany), 3 g/L yeast extract (Oxoid, United Kingdom), 30g/L gelatin (Oxoid, United Kingdom), 15 g/L agar (Scharlau, Barcelona, Spain), pH 7) supplemented with the desired concentration of each selected phytochemical. Plates with no added phytochemicals were used as negative controls. After incubation at 37 °C for 48 h, the

plates were swamped with a saturated solution of ammonium sulphate (VWR, Belgium). Gelatinase production was observed by a transparent halo formed around the cells. All tests were performed in duplicate with three repeats.

4.2.10.3 Proteases production

Production of proteases was accessed on Plate count agar (PCA; Oxoid, England) containing 1% skim milk powder (Merck, Germany), supplemented with the desired concentration of each selected phytochemical. Briefly, 10 µL of PA14 wild-type suspension (A_{600} = 0.1) were plated out on the aforementioned plates. Plates with no added phytochemicals were used as negative controls. The plates were incubated for 72 h at 30 °C. After the incubation time, the plates were swamped with 1 M HCl. Proteases production was observed by a transparent halo formed around the cells (Dogan and Boor 2003). All tests were performed in duplicate with three repeats.

4.2.10.4 Siderophores production

The detection of siderophores production was carried out in chrome azurol S (CAS; Fluka, Germany) agar plates, according to Schwyn and Neilands (1987). To prepare 1 L of CAS agar, 60.5 mg CAS were dissolved in 50 mL water and mixed with 10 mL iron(III) solution (1 mM $FeCl_3.6H_20$ (Panreac, USA), 10 mM HCl). This solution was gradually added to 72.9 mg hexadecyltrimethylammonium bromide (HDTMA; Merck, Germany) dissolved in 40 mL of water under stirring. The resultant dark blue liquid as well as basal agar medium (830 mL H_2O; 30 g 3-(N-morpholino)propanesulfonic acid (MOPS; Fisher Scientific, Belgium); 0.5 g NaCl; 0.3 g potassium phosphate (K_2PO_4;Chem-Lab, Belgium); 0.1 g ammonium chloride (NH_4Cl; Riedel-de Haën, Germany); 5 g L-asparagine (AppliChem, Germany); 15 g agar, pH 6.8) were autoclaved. After cooling to 50 °C, 20 mL glucose (50%) (Sigma-Aldrich, Portugal), the carbon source, as well as 100 mL of CAS indicator solution were finally added along the glass wall, with moderated agitation to prevent generation of foam. Each plate received 20 mL of CAS agar, supplemented with the desired concentration of each phytochemical. Ten microliters of *P. aeruginosa* PA14 wild-type suspension (A_{600} = 0.1) were plated out on the aforementioned plates. Plates with no added phytochemicals were used as negative controls. The plates were incubated at 37 °C, for 48 h. Siderophores production was observed by an orange halo formed around the cells. All tests were performed in duplicate with three repeats.

4.3 Results and discussion

4.3.1 Antimicrobial activity of selected phytochemicals

In order to know the antimicrobial and bactericidal activities of the compounds selected for this study, as well as to choose the suitable concentrations for QSI assays, the MIC and MBC were determined. Table 4 exhibits MIC and MBC values for the tested compounds against *P. aeruginosa* PA14 wild-type and biosensor *P. aeruginosa* PA14-R3. The MIC of the selected compounds ranged from 100 to > 1000 µg/mL and MBC values reported were always > 1000 µg/mL. Regarding phenolic compounds (RA, SA, and SYR), no inhibitory and bactericidal effects were observed. The alkaloid BE exhibited a MIC value of 1000 µg/mL. Regarding terpenoids, CITO exhibited a MIC of 400 µg/mL, whereas the inhibitory effects of the other tested terpenoids were not detected under 1000 µg/mL. PEITC did not show any inhibitory or bactericidal effects. FUR displayed the lowest MIC of all the compounds tested (100 µg/mL). Additionally, MIC and MBC values were the same for both bacteria. In general, FUR was the most effective compound assayed with respect to both inhibitory and bactericidal activities.

Table 4. MIC and MBC values of selected phytochemicals and derivatives against *P. aeruginosa* PA14 wild-type and biosensor PA14-R3.

Phytochemical	*P.aeruginosa* PA14		*P.aeruginosa* PA14-R3	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
RA	NA	NA	NA	NA
SA	NA	NA	NA	NA
SYR	NA	NA	NA	NA
BE	1000	NA	1000	NA
MYR	NA	NA	NA	NA
OCI	NA	NA	NA	NA
CITA	NA	NA	NA	NA
CITO	400	NA	400	NA
CARV	NA	NA	NA	NA
PEITC	NA	NA	NA	NA
FUR	100	200	100	200

(NA) – No activity, the MIC/MBC is higher than the maximum concentration tested (>1,000 µg/mL).

4.3.2 Phytochemicals mediated inhibition of the 3-oxo-C12-HSL-dependent QS system of *P. aeruginosa*

The *P. aeruginosa* PA14/PA14-R3 cocultivation system was used to screen the global effect of the phytochemicals presented in Table 4 on the *P. aeruginosa* 3-oxo-C12-HSL-

dependent QS system. Because this method revealed to be sensitive to DMSO, concentrations of this solvent equal to or lower than 6% (v/v) of the final volume of cell suspension were employed. However, solubility problems precluded the use of CAPE and its derivatives, PIPE, PIAC, and PEITC (MICs/MBCs not shown in Table 4).

According to Imperi et al. (2013), criteria used for selection of hit compounds were at least 50% of relative bioluminescence emission and a maximum of 20% in the reduction of cell growth with respect to the negative controls (cells with DMSO). The latter criterion was aimed at avoiding any unspecific effect of impaired growth on the QS response. The results of the high-throughput QSI screening are displayed in Figure 6. This screening assay allowed the identification of RA, SA, SYR, BE, and FUR as putative QSIs that reproducibly inhibited the QS response of the *P. aeruginosa* PA14/PA14-R3 cocultivation system without significantly affecting bacterial growth.

RA exhibited 50% of relative bioluminescence with no influence on bacterial growth for a concentration of 800 µg/mL. SA at 800 µg/mL presented a relative bioluminescence of 40% and an associated bacterial growth inhibition of 15%. For SYR, a minimum relative luminescence value of 40% and 10% of cell growth inhibition were achieved at a concentration of 400 µg/mL. These results confirm the potential of phenolic compounds to act as QS inhibitors against *P. aeruginosa*. Indeed, Rudrappa and Bais (2008) demonstrated that subinhibitory concentrations of curcumin, a diphenolic compound, were found to affect the QS system of this bacterium.

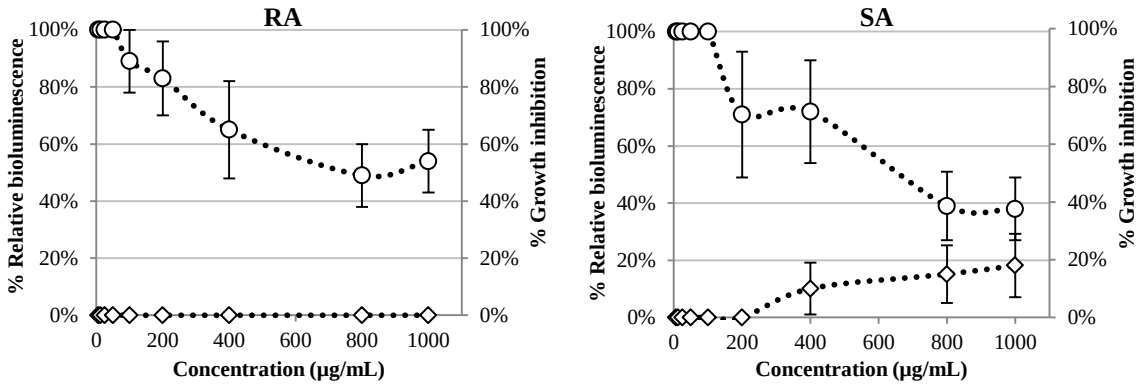
Regarding BE, a minimum relative bioluminescence value of 40% at 200 µg/mL was obtained, without affecting cell growth. FUR at 12.5 µg/mL exhibited a relative bioluminescence reduction of 60% with no interference on bacterial growth.

It is interesting to note that for RA and BE no bacterial growth inhibition was detected, at least at the minimum relative bioluminescence value reached. The same happened for FUR at concentrations up to 12.5 µg/mL. This is in accordance with the premise that any compound able to interfere with QS without affecting cell growth can be considered a promising inhibitor (Choo et al. 2006). Indeed, the selection of an effective QS inhibitor is based on its specify for a given QS regulator with no or little adverse effects on bacteria or host (Kalia 2013).

Concerning the results obtained for FUR, it should be noted that this compound is a synthetic nitrovinylfuran approved antibiotic with a broad antimicrobial spectrum that have the commercial name of Furvina® or G1 (MW 297). Moreover, FUR is also the active principle of an ointment (Dermofural®) used to treat a wide range of human dermatological infections (Fabbretti et al. 2012).

FUR was first developed in Cuba from sugarcane bagasse and in addition to its significant antimicrobial effect (values ranging from 0.5 to 32 mg/mL) comparable to that of some other antimicrobial agents currently in clinical use, it shows very low levels of toxicity. Despite its recognized potential, to my knowledge, its anti-QS activity was never tested. These findings, coupled with the fact that although several QS inhibitors have been identified to date, their clinical application remain far away, further highlights the importance of these results. Indeed, the identification of QS inhibitors among drugs already approved by clinical trial and used in human medicine is of valuable importance (Imperi et al. 2013). Therefore, the results achieved with FUR in this study are very promising.

The remaining phytochemicals tested were also capable of interfering with the QS response of the coculture (Figure 6). CITO and MYR achieved a minimum relative bioluminescence between 80 and 85% and an associated cell growth inhibition of 7% for 400 and 800 µg/mL, respectively. CITA and OCI at 800 µg/mL exhibited a relative bioluminescence of around 60% with growth inhibition percentages of 25 and 10%, respectively. CARV managed to reduce relative bioluminescence in about 25%, with also 25% of bacterial growth inhibition. Even though these essential oil components violate at least one of the criteria proposed by Imperi et al. (2013), as previously mentioned, the obtained results should not be devalued. It is clearly observed that the bacterium in presence of these compounds is capable to grow and to diminish its bioluminescence emission. Indeed, several studies have been reported the ability of essential oils to interfere with QS of *P. aeruginosa*, such as ferula and dorema oils (Sepahi et al. 2015), clove oil (Khan et al. 2009) and 6-gingerol (Kim et al. 2015).



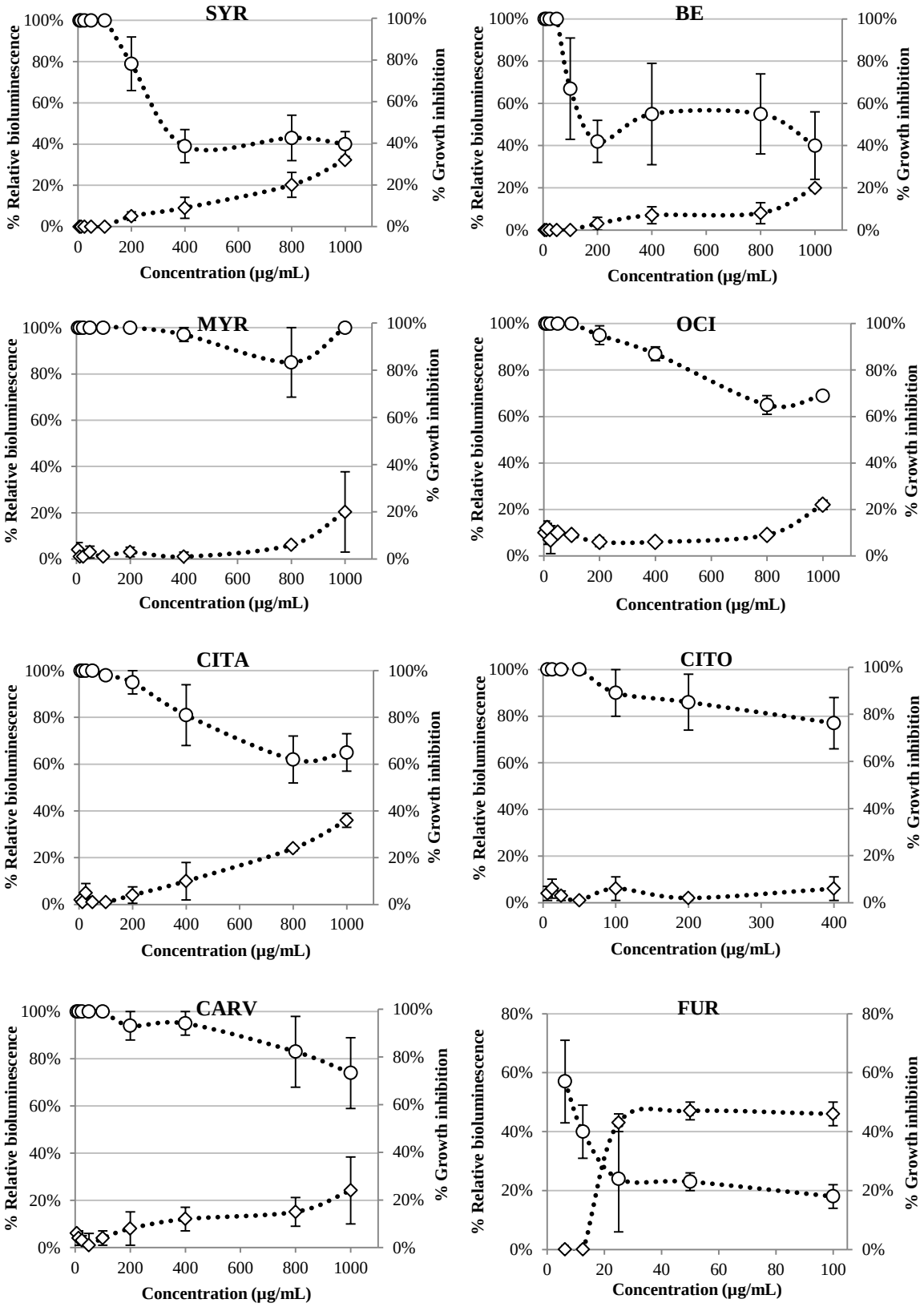


Figure 6. Effect of 10 selected phytochemicals (RA, SA, SYR, BE, MYR, OCI, CITA, CITO, CARV, FUR) on the 3-oxo-C12-HSL-dependent QS of *P. aeruginosa* using PA14/PA14-R3 cocultivation. The results are presented as percentages of relative bioluminescence (circle) versus growth inhibition (lozengue) as a function of sub-MIC concentrations of each phytochemical tested. Mean values ± SD for at least three replicates are illustrated.

These results show the ability of the phytochemicals tested to inhibit the *P. aeruginosa* 3-oxo-C12-HSL-dependent QS system. However, based only on the assays conducted it was not possible to infer about the exact mechanism of action of these compounds. Thus, additional studies to better understand if QSI is due to inhibition of the synthesis, detection or transport of the AIs are mandatory. In this context, Imperi et al. (2013) reported that niclosamide was able to diminish bioluminescence emission by *P. aeruginosa* PA14-R3 grown in the presence of exogenously added 3-oxo-C12-HSLs, suggesting that the QS-inhibitory activity of niclosamide is based on its ability to hamper the response of *P. aeruginosa* to the signal molecule rather than to inhibit its synthesis.

4.3.3 Preventive effect of selected phytochemicals on initial cell adhesion and biofilm formation

P. aeruginosa is responsible for chronic infections due to biofilm formation. Numerous studies using flow cell chambers for biofilm formation, together with confocal scanner electron microscopy observations, have shown that a proficient QS system is essential for optimal biofilm development (Kirisits and Parsek 2006). In this context, the ability of the above identified QS inhibitors (RA, SAR, SYR, BE, MYR, OCI, CITA, CITO, CARV, and FUR) to interfere with biofilm formation and aspects related with biofilm development such as initial adhesion (2 h) was tested. PEITC, an ITC, was also added to the list of tested compounds. Even though it was not screened for QSI activity, PEITC was found to possess anti-QS activity against *C.violaceum*, as stated by Borges et al. (2014a). In addition, other ITCs, namely iberin, are known to antagonize the QS system of *P. aeruginosa* (Jakobsen et al. 2012a). Thus, it is possible that PEITC can act as a QS inhibitor in the studied bacterium.

The effect of the initial inoculum cell density on the two factors mentioned above was also investigated. Therefore, two different initial inoculum concentrations (A_{600} = 0.04 and A_{600} = 0.004) were used in order to check if these would affect the prevention of initial adhesion and biofilm formation. Figures 7 to 10 and Tables 5 to 8 display the most interesting results.

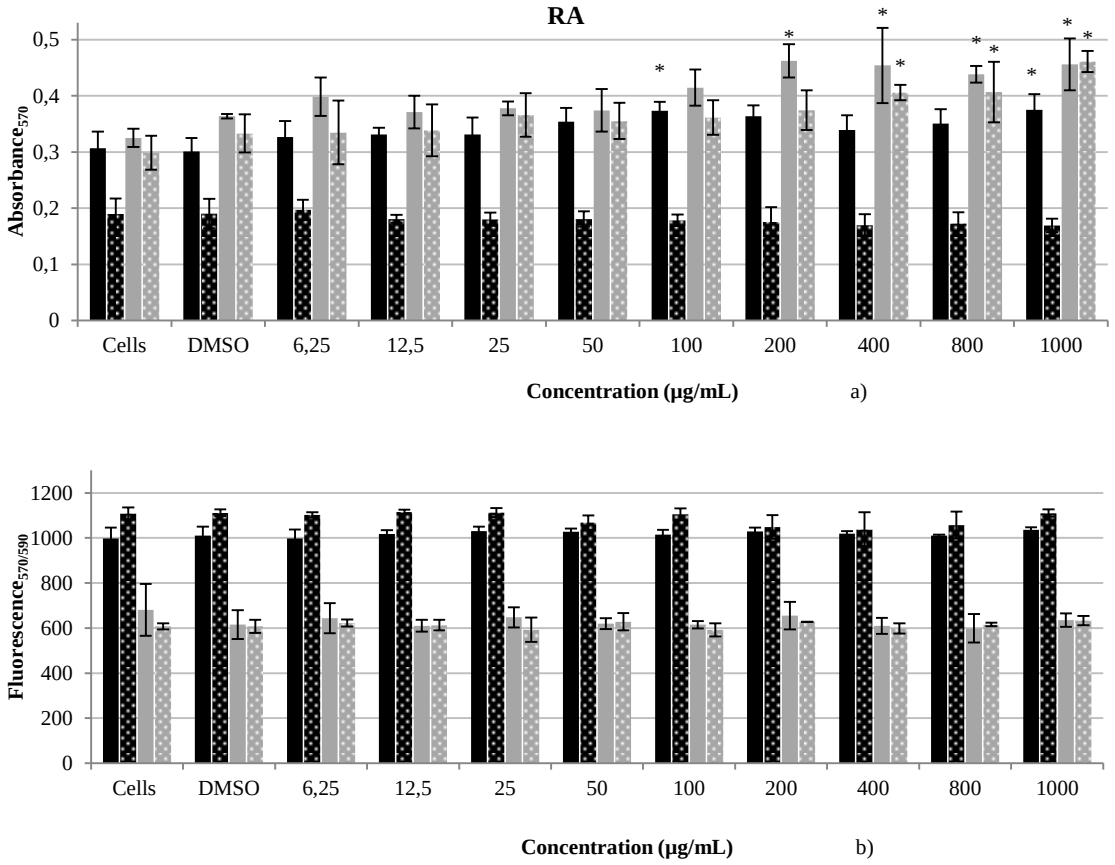


Figure 7. Preventive effects of RA at sub-MICs (6.25 to 1000 µg/mL) in the initial cell adhesion (2 h) (black) and in biofilm formation (grey) assessed in terms of biomass formation (a) and metabolic activity (b). Prior to experiments, PA14 wild-type cultures were adjusted to two different absorbance (A_{600}) values, 0.004 (no pattern) and 0.04 (pattern). Crystal violet (A_{570}) and alamar blue stainings ($Fluorescence_{570/590}$) were performed. Bars with * are statistically different from the control ($p < 0.05$).

Table 5. Percentages of adhered cells biomass and activity reduction by RA.

RA	Concentration (µg/mL)									Conditions	
	6.25	12.5	25	50	100	200	400	800	1000	Incubation time (h)	Starting A_{600}
% adhered cells biomass reduction	0	5	5	5	6	8	11	9	11	2h	0.04
% adhered cells activity reduction	1	0	0	4	1	6	7	5	0		

Note: Percentages are calculated with respect to the DMSO control.

Two hours-adhered cells treated with RA (Figure 7) and grown from an initial inoculum with an A_{600} of 0.04 showed a slight decrease in the adhered cells with the increase of the concentration whereas for the other conditions no preventive effects were detected. The highest value recorded was 11% at 400 and 1000 µg/mL (Table 5). Additionally, even though minor fluctuations in the viability of adhered biomass were detected, they were not regarded as statistically significant ($p > 0.05$). Comparing the results for both initial inoculum, it was

supposed that for a lower value of A_{600} more molecules of compound *per cell* would be available to perform their role as QS inhibitors. However, the results contradicted the expected. Hence, it is possible to infer that a higher number of initial cells potentiate the activity of RA as a QS inhibitor. Regarding the formation of 24 h old biofilms, RA did not seem to have any influence on biofilm prevention. Instead, it seems to potentiate biofilm development. Thus, it is possible that RA affects QS pathways responsible for the regulation of initial cell adhesion.

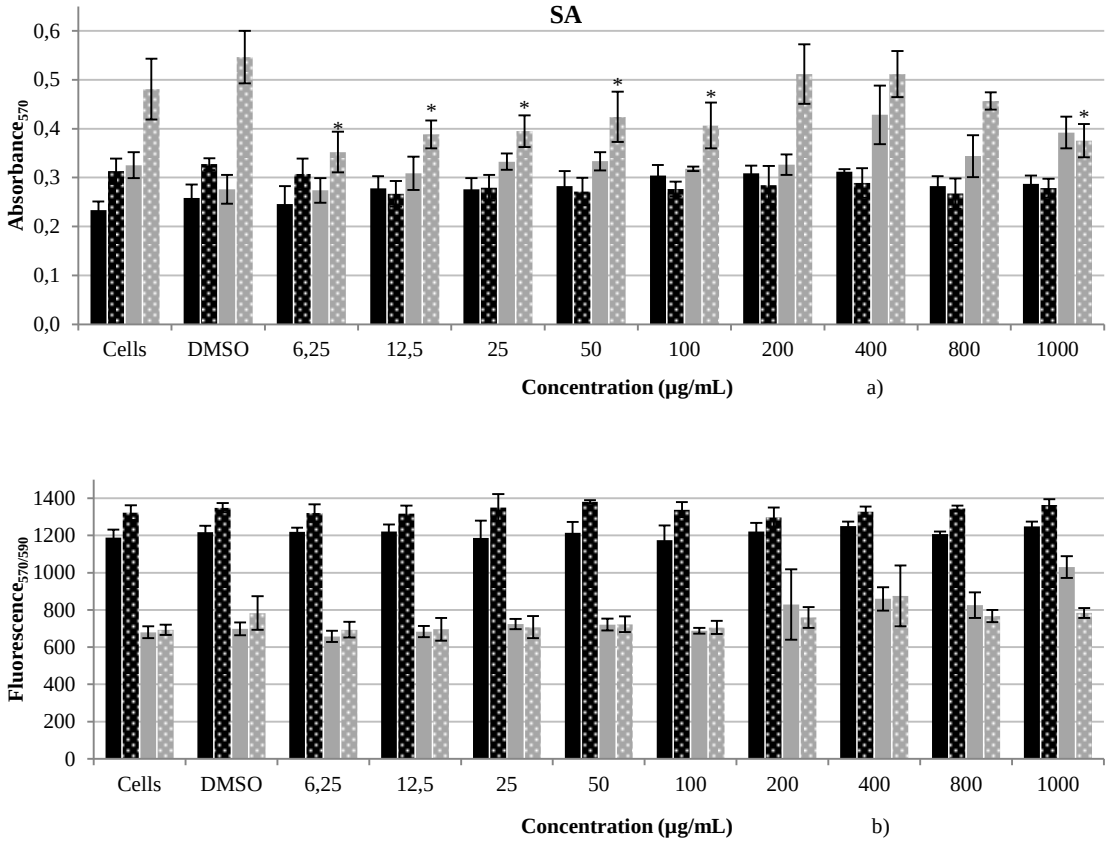


Figure 8. Preventive effect of SA at sub-MICs (6.25 to 1000 µg/mL) in the initial cell adhesion (2 h) (black) and in the biofilm formation (grey) assessed in terms of biomass formation (a) and metabolic activity (b). Prior to experiments, PA14 wild-type cultures were adjusted to two different absorbance (A_{600}) values, 0.004 (no pattern) and 0.04 (pattern). Crystal violet (A_{570}) and alamar blue stainings ($Fluorescence_{570/590}$) were performed. Bars with * are statistically different from the control ($p < 0.05$).

Table 6. Percentages of adhered cells biomass and activity reduction by SA.

SA	Concentration (µg/mL)									Conditions	
	6.25	12.5	25	50	100	200	400	800	1000	Incubation time (h)	Starting A_{600}
% adhered cells biomass reduction	6	18	15	17	16	13	12	18	15	2	0.04
% adhered cells activity reduction	2	2	0	0	1	4	2	0	0		
% adhered cells biomass reduction	26	29	28	22	26	6	6	16	31	24	0.04
% adhered cells activity reduction	11	11	10	8	10	3	0	2	0		

Note: Percentages are calculated with respect to the DMSO control.

The results for SA are displayed in Figure 8. Two hours-adhered cells treated with SA and grown from an initial inoculum with an A_{600} of 0.04 showed a slight decrease in the adhered biomass which was maintained more or less constant with the increase of the concentration. The highest value of adhered cell reduction recorded was 18% at 800 µg/mL (Table 6). Like RA, small fluctuations on cell viability were reported, however, they were considered statistically insignificant ($p > 0.05$). For the same initial inoculum density, 24 h-aged biofilms were also formed in the presence of SA. The results show a significant decrease on the adhered cells ($p < 0.05$), reaching a reduction of 31% for a concentration of 1000 µg/mL. At this concentration, no cell inactivation was detected. Hence, it is possible that SA could be more closely related with other aspects regarding biofilm development, such as production of EPS, rather than with initial cell adhesion. However, the results of cell adhesion should not be devalued. Indeed, Borges et al. (2013b) studied the influence of ferulic acid, structurally similar to SA, on *P. aeruginosa* adhesion and found that adhesion was less favorable when bacteria were exposed to the chemical. Again, a lower initial inoculum cell density is not favorable for SA to act as a QS inhibitor, since no reduction on the number of sessile cells was observed.

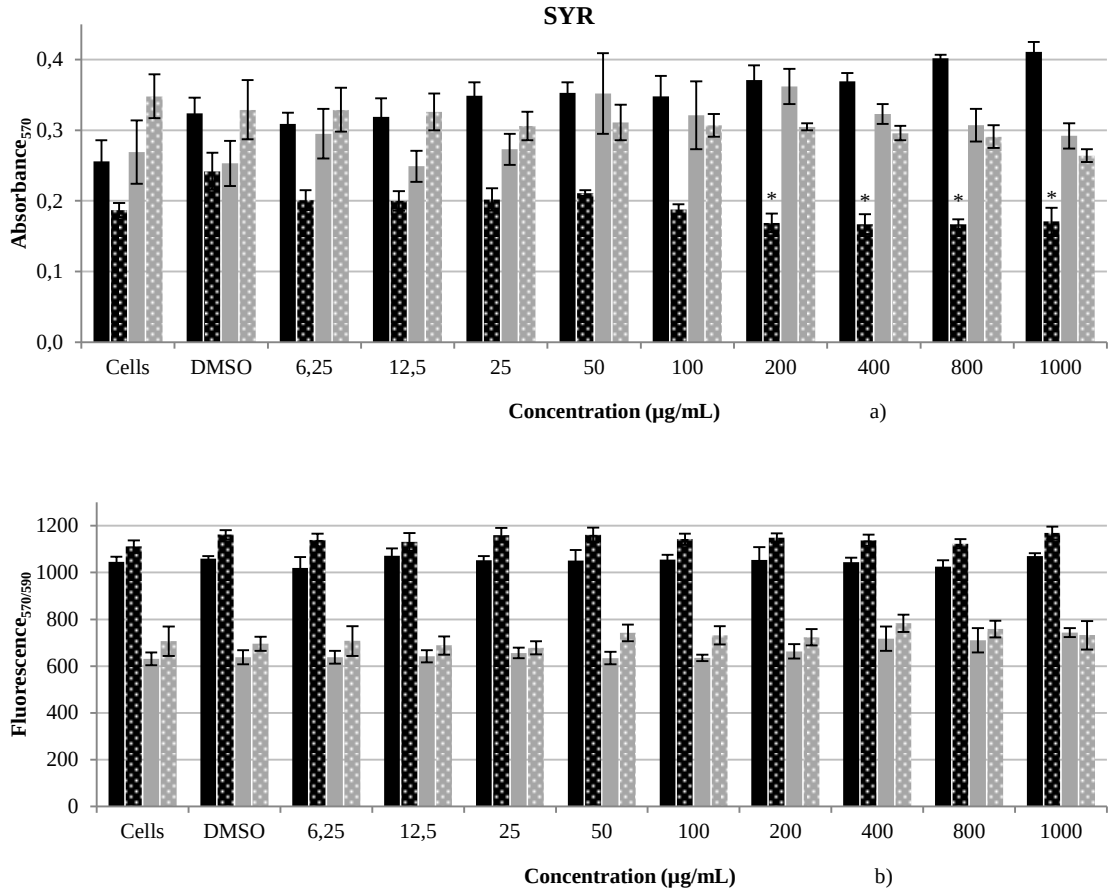


Figure 9. Preventive effect of SYR at sub-MICs (6.25 to 1000 µg/mL) in the initial cell adhesion (2 h) (black) and in the biofilm formation (grey) assessed in terms of biomass formation (a) and metabolic activity (b). Prior to experiments, PA14 wild-type cultures were adjusted to two different absorbance (A_{600}) values, 0.004 (no pattern) and 0.04 (pattern). Crystal violet (A_{570}) and alamar blue stainings ($Fluorescence_{570/590}$) were performed. Bars with * are statistically different from the control ($p < 0.05$).

Table 7. Percentages of adhered cells biomass and activity reduction by SYR.

SYR	Concentration (µg/mL)									Conditions	
	6.25	12.5	25	50	100	200	400	800	1000	Incubation time (h)	Starting A_{600}
% adhered cells biomass reduction	17	17	17	13	22	30	31	31	29	2	0.04
% adhered cells activity reduction	2	3	0	0	2	1	2	3	0		
% adhered cells biomass reduction	0	1	7	5	7	7	10	12	20	24	0.04
% adhered cells activity reduction	0	0	0	1	0	0	0	0	0		

Note: Percentages are calculated with respect to the DMSO control.

The results for SYR are displayed in Figure 9. Two hours-adhered cells treated with SYR and grown from an initial inoculum with an A_{600} of 0.04 showed a significant decrease in the adhered biomass with the increase of the concentration ($p < 0.05$). At 400 and 800 µg/mL, a decrease of 31% in the adhered cells was recorded, without affecting cell growth ($p > 0.05$) (Table 7). In the same way, preventive effect was observed by Borges et al. (2013b) with gallic acid, a phenolic acid structurally similar to SYR, against *P. aeruginosa* biofilms. Regarding the formation of 24 h old biofilms grown from an initial inoculum of 0.04, even though a decrease in the attached biomass has been reported, it was not considered statistically significant ($p > 0.05$). Again, a lower initial inoculum cell density is not favorable for SYR to act as a QS inhibitor, since no reduction on the number of attached cells is observed. Thus, it is likely to say that SYR may interfere with QS pathways responsible to coordinate initial cell adhesion. Again, a lower initial inoculum cell density is not favorable for SYR to act as a QS inhibitor, since no reduction on the number of attached cells is observed.

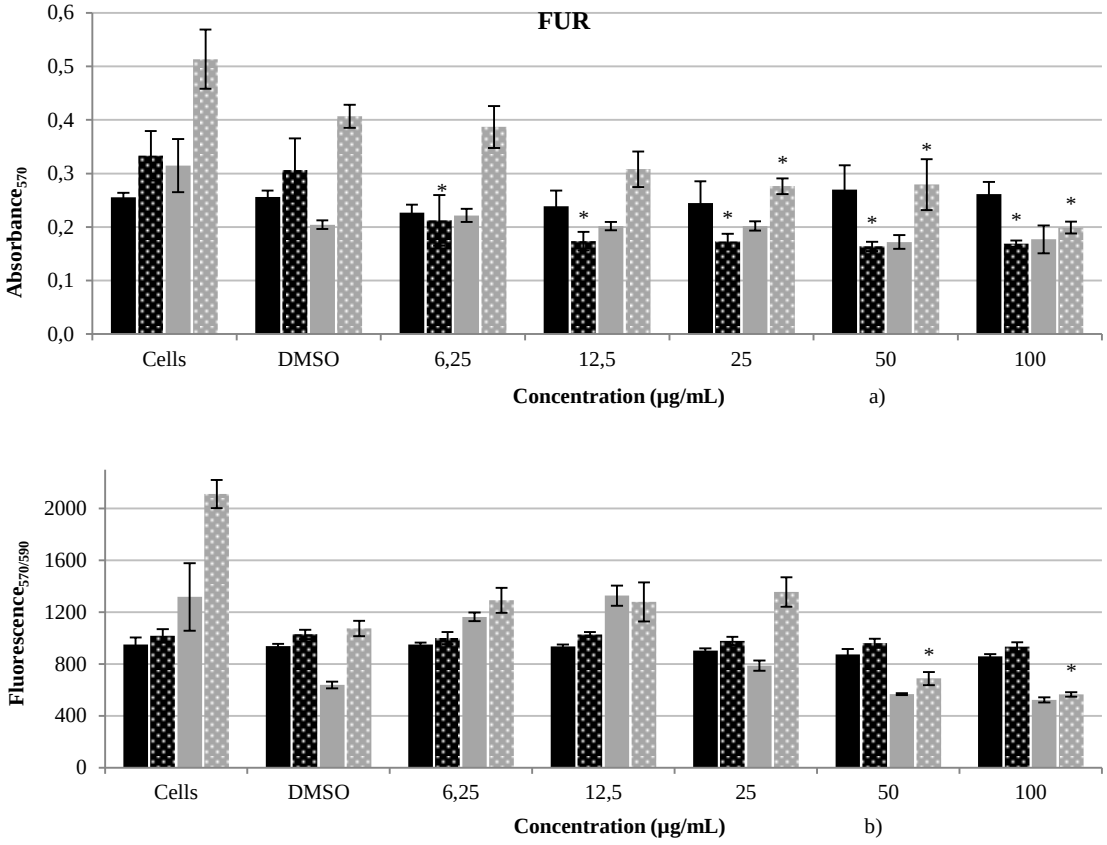


Figure 10. Preventive effect of FUR at sub-MICs (6.25 to 1000 µg/mL) in the initial cell adhesion (2 h) (black) and in the biofilm formation (grey) assessed in terms of biomass formation (a) and metabolic activity (b). Prior to experiments, PA14 wild-type cultures were adjusted to two different absorbance (A_{600}) values, 0.004 (no pattern) and 0.04 (pattern). Crystal violet (A_{570}) and alamar blue stainings ($Fluorescence_{570/590}$) were performed. Bars with * are statistically different from the control ($p < 0.05$).

Table 8. Percentages of adhered cells biomass and activity reduction by FUR.

FUR	Concentration (µg/mL)					Conditions	
	6.25	12.5	25	50	100	Incubation time (h)	Starting A_{600}
% adhered cells biomass reduction	31	43	44	47	45	2	0.04
% adhered cells activity reduction	3	0	5	7	9		
% adhered cells biomass reduction	0	1	1	16	13	24	0.004
% adhered cells activity reduction	0	0	0	11	18		
% adhered cells biomass reduction	5	24	32	31	51	24	0.04
% adhered cells activity reduction	0	0	0	36	47		

Note: Percentages are calculated with respect to the DMSO control.

The results for FUR are shown in Figure 10. A significant decrease in the percentage of adhered cells was observed for an incubation time of 2 h with the highest initial cell density (A_{600} = 0.04). The maximum value was recorded as 47 % at 50 µg/mL (Table 8). For these conditions, no significant cell inactivation was detected. Therefore, although FUR has had some preventive effect on initial attachment of cells, those that adhered continue metabolically active. The cell density in the initial inoculum seems to affect the initial attachment of cells, since at the lowest value no initial cell adhesion prevention was observed. Actually, it potentiates cell adhesion. Regarding the formation of 24 h old biofilms growing at an initial cell density of 0.004 (A_{600}), a decrease in cell adhesion was observed, with a maximum associated value of 16% at 50 µg/mL. Despite being statistically insignificant, slight oscillations in cell inactivation were observed. At the highest initial cell density (A_{600} = 0.04), 24 h old biofilms faced a significant decrease in cell attachment with an associated maximum value of 51% at 100 µg/mL. This concentration also presented a significant cell inactivation. However, at lower concentrations of FUR, significant cell attachment reduction also occurred, with an associated value of 32% at 25 µg/mL and this time with no cell inactivation. It is known that any compound able to interfere with QS without affecting cell growth can be considered a promising inhibitor (Choo et al. 2006). Thus, 25 µg/mL was considered the most promising concentration. Like cell adhesion for 2h, the effect of the initial inoculum cell density is contrary to the expected: less cell density in the initial inoculum leads to a low percentage of

adhered cells reduction. From these results, it may be said that FUR is involved with QS pathways responsible for both initial cell adhesion and biofilm differentiation/maturation.

4.3.4 Effect of QSI on the susceptibility of *P. aeruginosa* biofilms to ciprofloxacin

The emergence of multi-drug resistant bacteria is considered the major worldwide threat in the treatment of infectious diseases (Abreu et al. 2012). Moreover, treatment of infections associated to biofilms with existing approved therapies remains a significant medical challenge. It has been documented that phytochemicals with the ability to inhibit QS can be used along with antibiotics as adjuvants to increase the susceptibility of infecting bacteria (Borges et al. 2013a). Indeed, Brackman et al. (2011) demonstrated that QS inhibitors increase the susceptibility of bacterial biofilms to multiple types of antibiotics. In this context, the susceptibility of 24 h old biofilms grown in the presence of selected phytochemicals, to ciprofloxacin at 5 and 10 times its MIC was evaluated. The results are displayed in Figure 11 and Table 9.

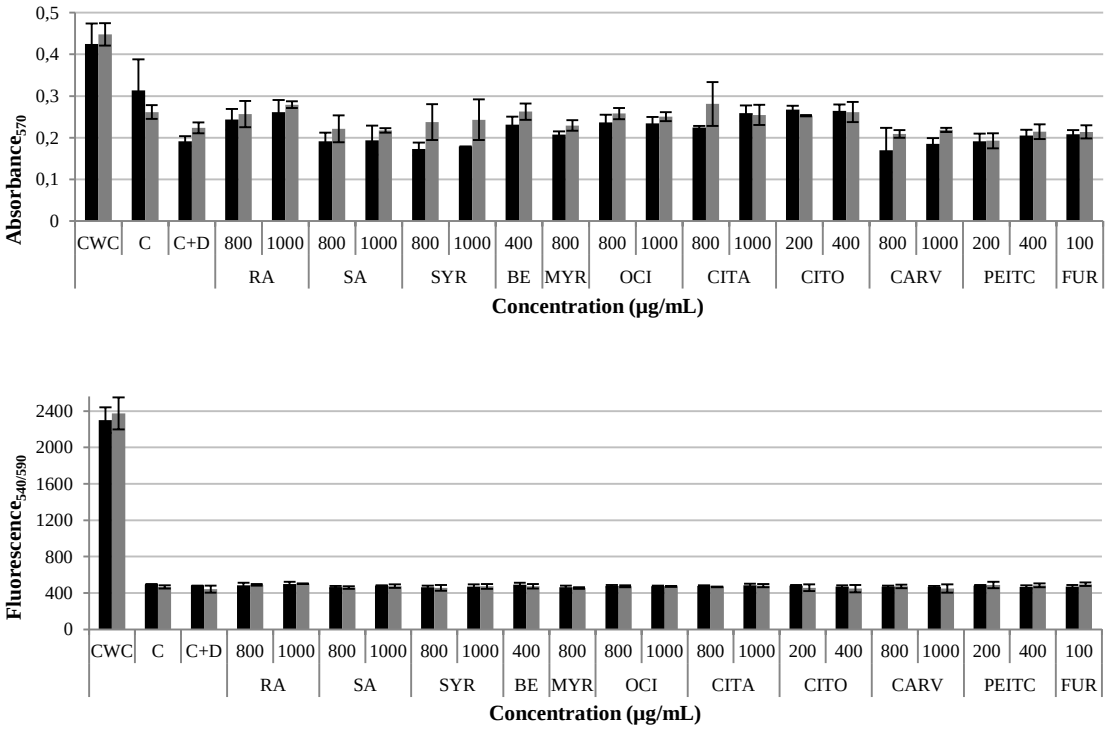


Figure 11. Susceptibility of 24h old *P. aeruginosa* biofilms formed in the presence of selected QS inhibitors to ciprofloxacin at 40 (black) and 80 (grey) µg/mL. For mass and metabolic activity quantification, crystal violet (A_{570}) and alamar blue stainings ($Fluorescence_{570/590}$) were performed, respectively. "CWC" means cell control without ciprofloxacin treatment, "C" means cell control with ciprofloxacin treatment and "C+D" means cell + DMSO control with ciprofloxacin treatment. All concentrations tested are equal to or below MICs. Mean values ± SD for at least three repeats are illustrated. Bars with * are statistically different from the C+D control ($p < 0.05$).

Table 9. Percentages of biofilm mass reduction and inactivation.

	Concentration (µg/mL)									Condition
	SA		SYR		CARV		PEITC		FUR	Ciprofloxacin concentration (µg/mL)
	800	1000	800	1000	800	1000	200	400	100	
% Biofilm mass reduction	N/A	N/A	10	6	11	3	N/A	N/A	N/A	40
% Biofilm inactivation	N/A	N/A	4	2	2	3	N/A	N/A	N/A	
% Biofilm mass reduction	1	3	N/A	N/A	6	2	14	4	4	80
% Biofilm inactivation	0	0	N/A	N/A	0	0	0	0	0	

Note: Percentages were calculated with respect to the DMSO control. (N/A) – Not applicable.

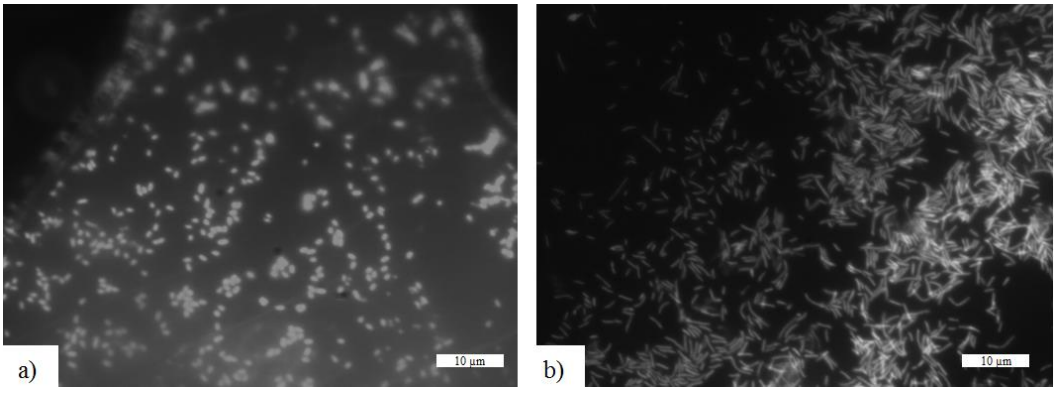
SA, SYR, CAR, PEITC, and FUR exhibited a slight decrease on biofilm formation ($p > 0.05$). In general, when comparing both tested concentrations of ciprofloxacin, its increase potentiates biofilm reduction. However, cell viability is more affected by a lower concentration of ciprofloxacin rather than a higher concentration, despite being statistically insignificant ($p > 0.005$). As previously mentioned, a synergism between QS inhibitors and antibiotics has been reported as a means to increase bacterial susceptibility. However, as shown in Figure 10, biofilm formation in the presence of most of the phytochemicals does not potentiate the antimicrobial activity of the antibiotic in study. Instead, the potency of ciprofloxacin is enhanced, as demonstrated by the absorbance values. Ciprofloxacin is a broad-spectrum antibiotic belonging to fluoroquinolone class that is active against both Gram-positive and Gram-negative bacteria. As ciprofloxacin promoted a significant mass reduction and viability of biofilm cells, other antibiotics less effective should be also tested in future work.

4.3.5 Effect of phytochemicals on biofilm structure

Direct microscopic observations of biofilms after exposure to phytochemicals are known to provide valuable information of the action of phytochemicals on biofilm development based mainly on their structural organization. Therefore, epifluorescence microscopy analyses were performed using DAPI stain. Problems regarding samples' preparation precluded the microscopic visualization of some of them.

The results show distinct biofilm architectures where bacteria grown in the absence or in the presence of different phytochemicals exhibited distinct behaviors. In Figure 12 (a), an untreated biofilm is exhibited. It is possible to observe that biofilms cells were embedded within

the EPS matrix. The EPS molecules are considered the major factor affecting biofilm architecture and maturation. They provide the mechanical stability of biofilms which allows the building of structured and complex communities, within which can occur extensive cellular differentiation. In addition, EPS production is often QS regulated, with several aspects of biofilm dynamics including, heterogeneity, architecture, stress resistance, maintenance and sloughing being mediated by signaling molecules of the type of AHLs (Borges et al. 2015). Herein, it was possible to observe that some phytochemicals reduced significantly the production of EPS as well as they caused disruption of the biofilm architecture (Figure 12 (d) and 12 (g)). Little portions of EPS around some cells were observed for biofilms formed in the presence of MYR and CARV (Figure 12 (f) and 12 (h)). For RA, SA, BE, PEITC and FUR (Figure 12 (b), (c), (e), (i) and (j)) no EPS was visually detected. In this context, it is possible that this putative reduction and/or absence of EPS can be due to the QSI potential of the selected phytochemicals, as previously demonstrated in section 4.3.2. Also, some of these results can be corroborated by the biofilm assays in section 4.3.3, where SA, SYR and FUR demonstrated the capacity to interfere with 24 h old biofilm formation. Comparing all the different biofilms in the presence of the tested phytochemicals, it is possible to observe that their organization and cellular density differs. CITO-treated biofilm exhibited the highest degree of cell density, whereas in FUR-treated biofilm cells are very disperse. Besides, it was observed that in general phytochemicals changed also the cell form, making them more elongated. This was particularly evident with RA and MYR, as it is possible to see in Figure 12 (b) and 12 (f).



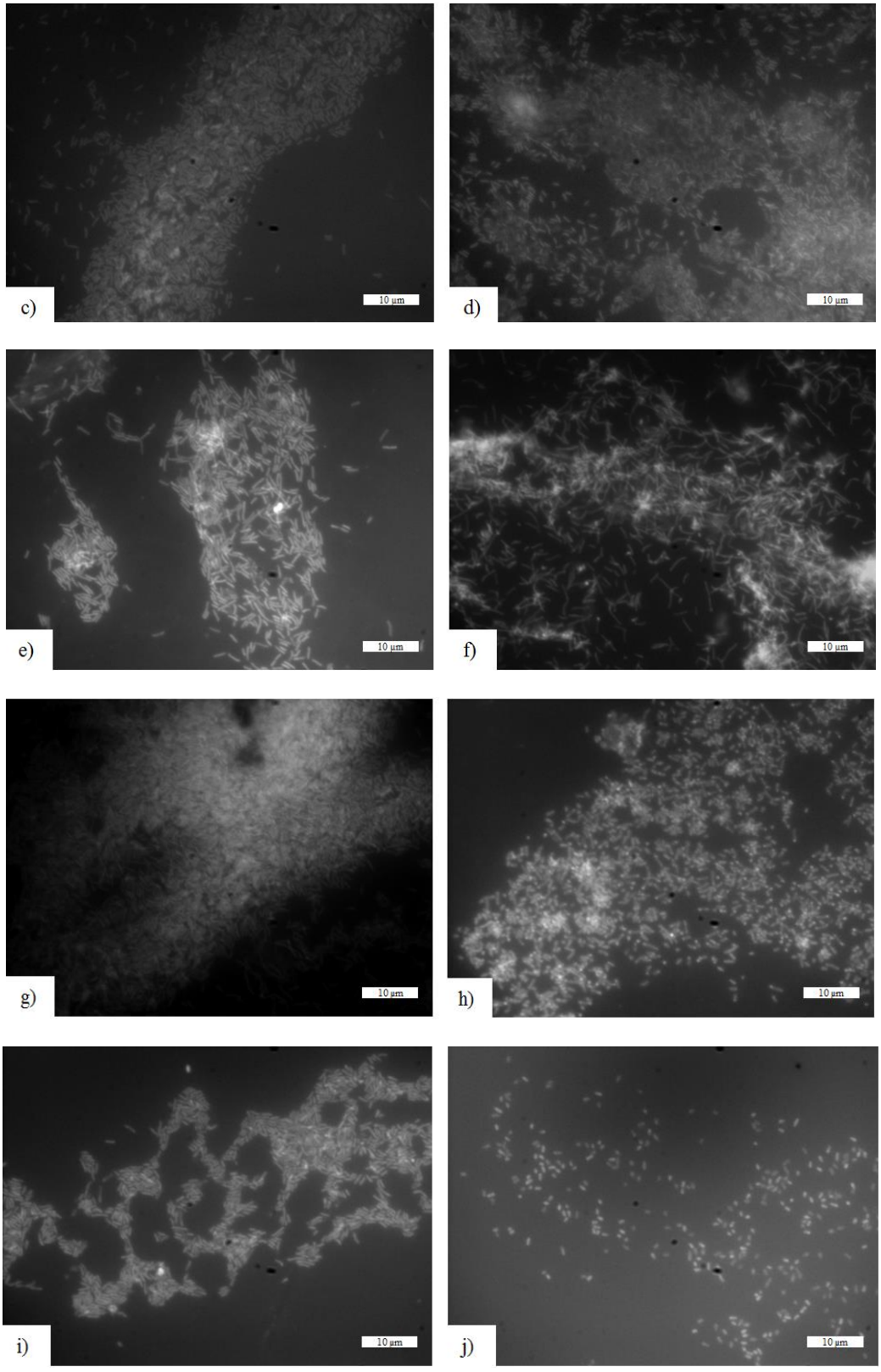


Figure 12. Epifluorescence microscopic images of DAPI stained bacterial biofilms grown in the absence and presence of different phytochemicals: (a) negative control; (b) RA at 800 µg/mL; (c) SA at 1000 µg/mL; (d) SYR at 800 µg/mL; (e) BE at 400 µg/mL; (f) MYR at 800 µg/mL; (g) CITO at 800 µg/mL; (h) CARV at 1000 µg/mL; (i) PEITC at 400 µg/mL; (j) FUR at 100 µg/mL.

4.3.6 Effect of QSI on virulence factors production

P. aeruginosa produces a wide array of virulence factors and evades the immune system by a great variety of adaptive mechanisms. Treatment of *P. aeruginosa* infection is difficult to achieve due to the problem of multidrug resistance and due to the production of several virulence factors including pyocyanin, siderophores and proteases, among others that contribute to its pathogenesis (El-Mowafy et al. 2014). The production of many of the key virulence factors is controlled by QS. Thus, the disruption of this process by chemical interference is becoming a topic of increasing interest in the pharmaceutical industry and academia (Hansen et al. 2015). Most studies of QS in *P. aeruginosa* have been focused on its role in pathogenicity. In this context, the effect of QS inhibitors on the inhibition of virulence factors production controlled by QS such as pyocyanin was studied. Pyocyanin, a redox-active small molecule, is one key virulence factor produced by *P. aeruginosa* at high cell density in response to the *Las* and *Rh1* AHL signal. This phenazine derivative in addition to acting as a terminal signal in QS pathway has the ability to maintain *P. aeruginosa* redox balance, especially under low oxygen conditions, and also protect it from reactive oxygen species (Miller et al. 2015). Besides, pyocyanin induces neutrophil apoptosis, inflammatory response and neutrophil-mediated tissue damage.

The efficacy of the compounds at reducing pyocyanin levels was calculated with respect to cell suspension without treatment. In general, the two highest subinhibitory concentrations of phytochemicals were chosen for this assay. However, viscosity problems associated with some of the samples after pyocyanin extraction precluded the analysis of FUR and BE, as well as some concentrations of the other phytochemicals. The results of the pyocyanin assay are presented in Figure 13.

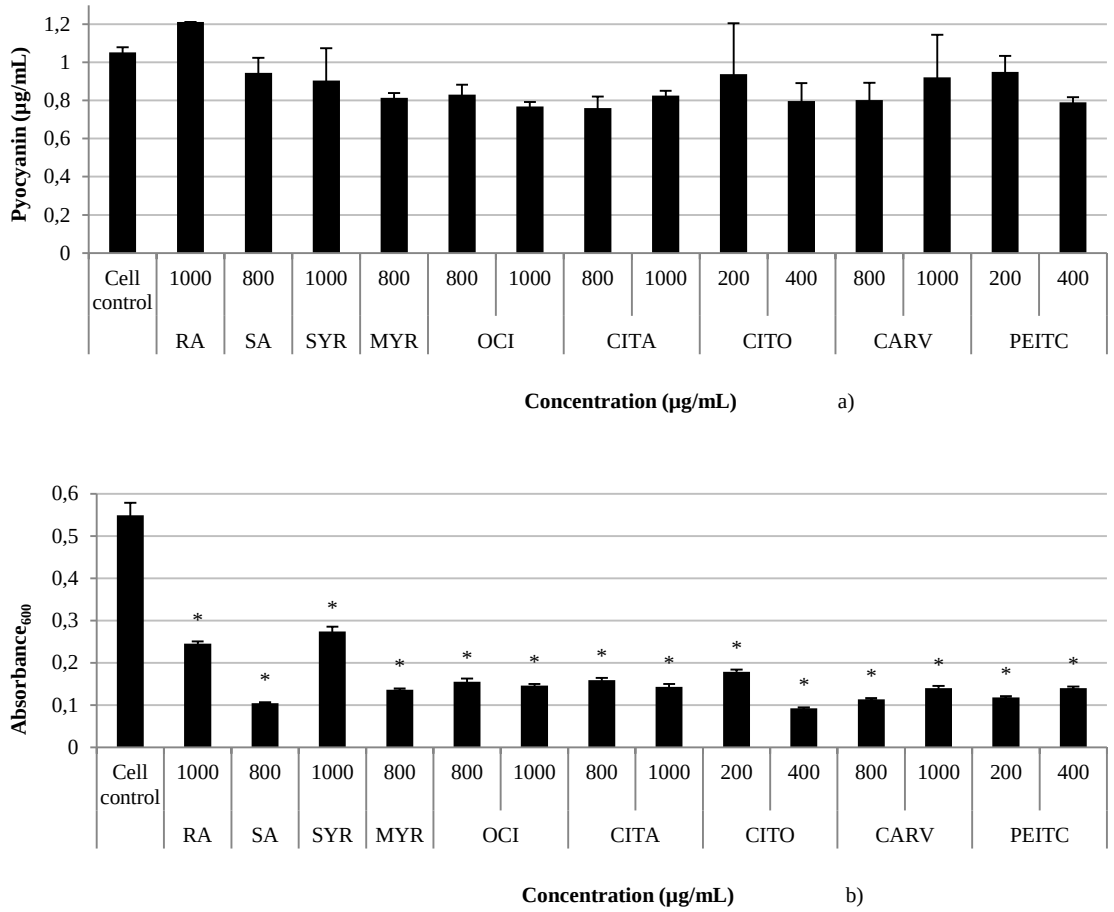


Figure 13. Influence of selected phytochemicals on pyocyanin production (pyocyanin µg/mL) (a) and in the cell growth (b) (A_{600}). In all cases, 10% DMSO (v/v) is present. The levels of pyocyanin were measured in cell-free supernatants from cultures of the wild-type strain Pa14. Mean values ± SD for at least three replicates are illustrated. Bars with * are statistically different from the cell control ($p < 0.05$).

In general, the tested compounds demonstrated a slight decrease in the quantity of pyocyanin extracted ($p > 0.05$). However, RA breaks the mould since it enhances pyocyanin production compared to the control with only cells. Similar behavior was obtained by Ahmad et al. (2015) with essential oil components such as β-pinene, (+)-limonene, (+)-borneo and (+)-carvone. Among all compounds assayed in the present work, both terpenoids OCI (1000 µg/mL) and CITA (800 µg/mL) were the phytochemicals with better performance. The growth of *P. aeruginosa* in the presence of phytochemicals seems to affect cell growth significantly ($p < 0.05$). Cugini et al. (2007) observed that farnesol (a common sesquiterpene) or compounds with related moieties caused a decrease in the production of the PQS and the PQS-controlled virulence factor (pyocyanin) by *P. aeruginosa*, without affecting the overall growth kinetics. Comparing farnesol, OCI and CITA structures, it is possible to see that the length and the number of double bonds in the chain affect the bioactivity of terpenes. Even though the results obtained have not been as significant as expected, they should not be devalued, and future work

at molecular level should be conducted to investigate the effects of the tested compounds in the down-regulation of gene expression related with pyocyanin production. Besides, structural modification could be one valuable option to attain the desired activity. Indeed, a lot of phytochemicals and derivatives have been reported to have influence on pyocyanin production in *P. aeruginosa*, from curcumin to eriodictyol (Rudrappa and Bais 2008; Vandeputte et al. 2011).

P. aeruginosa proteases are considered important virulence factors which damage host tissues and interfere with host antibacterial defense mechanisms (Ołdak and Elzbieta 2005). To address the question whether phytochemicals alter the production of extracellular proteases by *P. aeruginosa*, the proteolytic activity was assessed in the presence of the tested compound and compared with negative controls (plates without phytochemical). For this a simple qualitative plate assay based on the measurement of the clear halo (hydrolysis zones) produced around the bacterial drops, which permit detect the presence of extracellular proteases directly in the culture medium (agar plates), was employed. Skimmed milk agar plate assays (using skim milk as substrate) allow principally for qualitative determinations of total protease activity. In the same way the gelatin hydrolysis was used to detect activity of proteolytic enzyme, gelatinase. These methods are based on the hydrolysis of substrate for detection of proteases. Thus, the hydrolysis zone produced on both, skim milk/gelatin agar plates could be related to the amount of protease produced by *P. aeruginosa*.

Data regarding gelatinase, proteases and siderophores assays are displayed in Table 9. *P. aeruginosa* produced hydrolysis halos of 37 and 49 mm for gelatinase and protease, respectively. Reduction in the amount of gelatinase produced reflected by a decrease of the clear halo, from about 4 mm, was observed with OCI at both concentrations tested. Similar result was observed for FUR at 100 µg/mL. FUR also decreased proteases production in 12 mm. A reduction of the zones of proteolysis was too verified with PEITC (400 µg/mL) as well with RA (1000 µg/mL) in 7 mm and in 4 mm, respectively. Besides, it was found that the produced halo remains constant along the incubation time, whereas for the control (plates without phytochemical) an increase was observed. So, although a significant decrease has not been achieved it is possible speculate that the bacterium in study does not continue to produce this enzyme when the referred compounds are present in the medium. In a study performed by Stehling et al. (2008) with clinical strains of *P. aeruginosa* (from patients with cystic fibrosis and with extra-pulmonary infections), gelatinase production was different between non-mucoid and mucoid (generally more virulent) strains, being produced more insensible in these later. Protease-deficient strains are generally less virulent than protease producers in burned mouse models (Holder and Haidaris 1979). Therefore, protease production, including gelatinase should be

taken carefully as a virulence marker when pathogenicity characteristics are studied since they could be necessary for the basic maintenance of the colonization process (mediate tissue damage during *P. aeruginosa* infection in diverse body sites) and therefore the survival of bacteria in the host's tissues. In this way the results attained in the present work can be considered of potential interest.

Table 10. Halos of the gelatinase, proteases and siderophores assays.

Phytochemical	Concentration (µg/mL)	Halo (mm)		
		Gelatinase	Proteases	Siderophores
Control	-	37	49	18
RA	800	N/A	48	N/A
	1000	37	45	N/A
SA	800	38	47	19
	1000	38	48	N/A
SYR	800	38	49	18
	1000	38	49	16
BE	400	37	48	13
MYR	800	38	48	19
OCI	800	33	48	16
	1000	33	46	18
CITA	800	N/A	48	19
	1000	35	48	N/A
CITO	200	37	47	N/A
	400	N/A	49	N/A
CARV	800	37	49	N/A
	1000	35	46	18
PEITC	200	35	46	18
	400	37	42	16
FUR	100	33	37	13

Note: Halos include the diameter of the cells. (N/A) - Not applicable.

Regarding siderophores, both BE and FUR achieved a production decrease of about 5 mm. Any factor influencing the siderophore secretion (pyoverdin and pyochelin) by *P. aeruginosa* would greatly influence the efficacy of this opportunistic pathogen in promoting disease, since they are important for both bacterial virulence and biofilm development. These iron-chelating molecules compete with mammalian cells for iron, and when successful

sequestration occurs, they starve the host tissues (Lamont et al. 2002). In this sense, the results obtained deserve special attention.

Together the results obtained confirm the potential of some of the tested phytochemicals to interfere with the production of virulence factors in a QS-dependent manner. In support or our findings, a lot of phytochemicals were already identified as compounds that directly influence the production of *P. aeruginosa* virulence factors, such as catechin, curcumin, naringerin, phenylacetic acid and taxifolin, among others (Rudrappa and Bais 2008; Yang et al. 2009; Vandeputte et al. 2010; Vandeputte et al. 2011; Musthafa et al. 2012).

4.4 Conclusions

The present study has proved the role of all the selected phytochemicals in the attenuation of *P. aeruginosa* virulence and pathogenicity, by interfering with at least one of the different processes depending on cell density and regulated by QS (i.e. prevention of initial adhesion and biofilm formation and expression of virulence factors), therefore, altering the success of these pathogens in the colonization of a sensitive host and the development of an infectious process.

To date, no documented information about the potential QSI activity of these compounds was reported. Interestingly, FUR is commercially sold as a broad-spectrum antibiotic against bacteria and fungi. Indeed, the identification of QS inhibitors among drugs already approved by clinical trial and commercially available is of valuable importance. Therefore, the results achieved with FUR in this study are very promising.

CHAPTER 5

Concluding remarks and perspectives for future research

5.1 General conclusions

QSI is an effective antivirulence strategy to prevent biofilm infections caused by different pathogenic bacteria. In addition, secondary products from plants have proven to be outstanding compounds with many beneficial biological properties. Therefore, phytochemicals with QSI activity are promising tools for the treatment of bacterial infections in an era where availability of effective antibiotics is no longer guaranteed.

With the proposed work it was possible to highlight the potential of phytochemicals and derivatives as a green and sustainable source of new anti-QS products. This study also emphasized the potential of these compounds to interfere with QS-controlled phenotypes, namely bacterial adhesion, biofilm formation and production of virulence factors.

Among fourteen phytochemicals and five derivatives tested, CABn, PIAC, CITO, CARV and FUR revealed QSI activity against *C. violaceum* and CITO and CARV showed their ability to interfere with the AHL synthesis. RA, SA, SYR, BE, MYR, OCI, CITA, CITO, CARV and FUR were found to affect the 3-oxo-C12-HSL-dependent QS system of *P. aeruginosa.*

This work provided more information about the QS-dependent phenotypes of *P. aeruginosa* that are affected by the claimed QS inhibitors. RA, SA, SYR and FUR were able to interfere with the initial cell adhesion and biofilm formation of *P. aeruginosa*. Additionally, SA, SYR and FUR, as well as CARV and PEITC demonstrated a capacity to potentiate susceptibility of *P. aeruginosa* biofilms to ciprofloxacin. Moreover, light microscopic analysis of biofilm (grown in the presence of the putative QS inhibitors) structures revealed the ability of these compounds to affect EPS production. Finally, a reduction in the production of virulence factors was achieved by the tested compounds. All the phytochemicals were able to diminish pyocyanin production, especially OCI and CITA. OCI also had the capacity to interfere with gelatinase production. PEITC and RA were successful in decreasing the levels of protease production and BE managed to reduce siderophores production. FUR was able to interfere with all the virulence factors studied.

Despite the need to perform cytotoxicity assays in order to ascertain the potential use of the QS inhibitors as safe drugs, the overall results suggest that the tested compounds have potential to be used for therapeutic purposes. Additionally, all the studied compounds possess a partition coefficient (log*P*) ≤ 5 (Table A1 in Appendix), which according to Lipinski's "rule of five" is one of the requisites for these molecules to be considered as drug-like compounds.

Interestingly, FUR is a clinically approved antibiotic, available in the market under the name of Furvina®. Since this drug is already in the market, its unsuitability for use in humans is not regarded as an obstacle. In addition, it has been stated that Food and Drug Administration (FDA)-approved drugs may be endowed with antivirulence properties that are worthy of exploration. Hence, these results obtained for FUR should be considered of high relevance.

5.2 Future work

Based on the experimental work and the results obtained in this thesis, there are several perspectives for future work that would provide more interesting information about the topics addressed in this study. However, due to time constraints some tests were not possible to perform.

Even though several studies claim QSI activity of phytochemicals and derivatives, many of them rely heavily on the inhibition of QS-regulated phenotypes in biosensor strains. Hence, despite years of extensive and relevant research, some authors state that this kind of experiments is prone to bias, and adequate control experiments are essential to confirm the reliability of those promising compounds as QS inhibitors.

In this context, it should be verified if the putative QS inhibitors have no effect on the reporter phenotype when it is independent of QS. This can be achieved by the use of a biosensor strain in which the reporter phenotype is under control of a constitutive or inducible promoter. In addition, the determination of the exact mechanism of action (synthesis, detection or transport of the AIs across the cell envelope) of the QS inhibitors in the QS system is also recommended. For instance, to search for interference with AHL detection in *P. aeruginosa*, exogenously added 3-oxo-C12-HSLs can be provided to the environment.

Moreover, it should be accessed the impact of the claimed QS inhibitors in other phenotypes that are QS-dependent, such as hydrogen cyanide, pyoverdin and rhamnolipid biosynthesis in *P. aeruginosa.*

Transcriptomic analyses should also be carried out in order to determine whether the set of genes that are affected by the putative QS inhibitors match the set of genes that are under the

QS control. The use of QS inhibition selection systems to corroborate the inhibition potential of the putative QS inhibitors is also another interesting idea. These systems allow the identification of AHL QS inhibitors and growth inhibition at the same time, thereby avoiding false positives due to a growth inhibitory effect.

More aspects about the exact mechanism that QS inhibitors trigger in biofilm formation are required. For instance, it would be important to evaluate the interference of QS inhibitors in bacterial motility and EPS production.

Considering that most investigation involving biofilms have been performed using *in vitro* studies and the lack of direct observation of biofilms in their environment, the significance of biofilms is not well understood. Hence, more studies using QS inhibitors in *in vivo* models (e.g. *Galleria mellonella* insect model of infection) can provide new important data that can help in biofilm regulation.

In addition, in order to infer about the safety of possible QS inhibitors for human health, cytotoxicity tests are required.

The results obtained during this study showed that sometimes the high concentrations of phytochemicals necessary to promote significant anti-QS effects overcome those clinically acceptable. Additionally, since a compound that is considered a good QS inhibitor does not suppress cell growth, a perfect scenario would be that of QSI activity without cell growth inhibition. In this context, one possible strategy is to improve the potency and/or selectivity of phytochemicals by tailored structural modification. Indeed, computational techniques can assist in searching the drug target and in the optimization of lead compounds more quickly.

It is expected that the inclusion of some of these experiments in studies aiming to discover/enhance QS inhibitors should result in more reliable compounds that hopefully will become true QS inhibitors in a near future.

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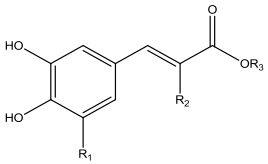
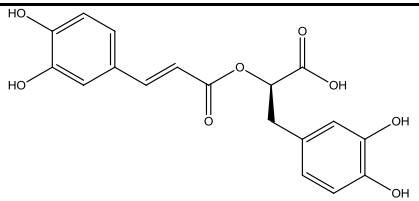
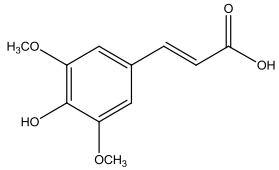
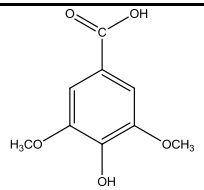
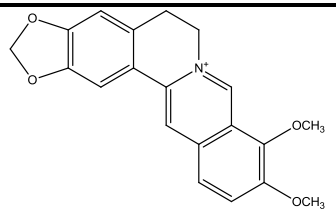
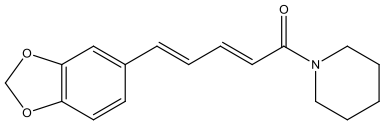
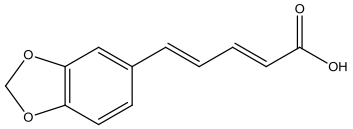
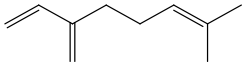
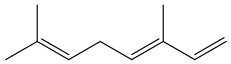
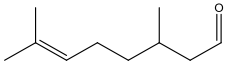
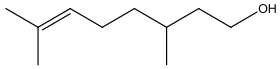
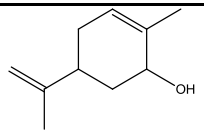
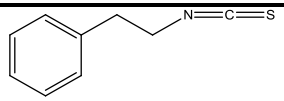
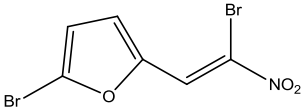
APPENDIX

Table A1. Selected phytochemicals, their chemical class and structure, partition coefficient (log *P*), dietary source and biological properties.

Chemical class	Phytochemical	Abbr.	Chemical structure	R_1	R_2	R_3	log*P*	Dietary source	Biological properties	References
Phenolic	Caffeic acid phenethyl ester	CAPE		H	H	CH_2CH_2Ph	3.43	Propolis derived from the bark of conifer trees	Antiviral, antiinflammatory antioxidant, anticancer, immunomodulatory	(Choi et al. 2015; Pittala et al. 2015)
	Caffeic acid phenethyl ester derivatives	CABn		H	H	Bn	3.15	N/A	N/A	N/A
		NBn		NO_2	H	Bn	2.92			
		CNBn		NO_2	CN	Bn	2.26			
		NCAPE		NO_2	H	CH_2CH_2Ph	3.37			
		CNCAPE		NO_2	CN	CH_2CH_2Ph	2.72			
	Rosmarinic acid	RA		N/A	N/A	N/A	2.07	*Lamiaceae* (mint) family	Antioxidant, antiinflammatory, anticancer, antimicrobial	(Ramalho et al. 2014)
Phenolic acid	Sinapic acid (hydroxycinnamic acid)	SA		N/A	N/A	N/A	1.29	Rye, fruits, vegetables	Antioxidant, antihyperglycemic, antiinflammatory, antibacterial	(Moura et al. 2013; Silambarasan et al. 2015)
	Syringic acid (hydroxybenzoic acid)	SYR		N/A	N/A	N/A	0.95	*Alpinia calcarata Roscoe*	Antioxidant, antiproliferative, anticancer, antiendotoxic.	(Srinivasan et al. 2014)

Alkaloyd	Berberine (isoquinoline alkaloid)	BE		N/A	N/A	N/A	-0.77 *	*Berberis aristata*, *Berberis aquifolium*	Antibacterial, antihypertensive, antiinflammatory, antidiabetic, antihyperlipidemic, anticancer	(Tsang et al. 2015; Wu et al. 2015)
	Piperine	PIPE		N/A	N/A	N/A	2.78	Black pepper, (*Piper nigrum*) long pepper (*Piper longum)*	Antidepressant, hepatoprotective, antimetastatic, antithyroid, immunomodulatory, antitumor	(Gupta et al. 2015; Hu et al. 2015)
ABA	Piperic acid	PIAC		N/A	N/A	N/A	2.23	N/A	Antimicrobial, antioxidant	(Zarai et al. 2013)
Terpenoid	Myrcene	MYR		N/A	N/A	N/A	3.33	Wormwood, bay leaf, lemongrass	Analgesic, antiinflammatory, anticancer, antigout, antibacterial	(Lorenzetti et al. 1991; Russo 2011; Olorunnisola et al. 2014)
	Ocimene	OCI		N/A	N/A	N/A	3.28	*Ocimum basilicum*	Antibacterial, antioxidant, anticancer	(Shafaghat 2009; Mahmoud 2013)
	Citronellal	CITA		N/A	N/A	N/A	2.35	Citronella, kaffir lime	Antioxidant, antibacterial, antinociceptive	(Victoria et al. 2014)
	Citronellol	CITO		N/A	N/A	N/A	2.82	Citronella, geranium	Antifungal, anticonvulsant	(Viollon and Chaumont 1994; de Sousa et al. 2006)
	Carveol	CARV		N/A	N/A	N/A	1.92	Spearmint	Anticancer, antimicrobial	(Crowell et al. 1992; Hussain et al. 2010)

GHP	2-phenylethyl isothiocyanate	PEITC		N/A	N/A	N/A	3.47	Watercress and *Brassica* species	Anticarcinogenic, QS inhibitor	(Hong and Kim 2008; Borges et al. 2014b)
FRCD	(Z)-2-bromo-5-(2-bromo-2-nitrovinyl)furan	FUR		N/A	N/A	N/A	3.00 *	Sugarcane bagasse	Antibacterial, antifungal	(Blondeau et al. 1999)

(*) – Clog*P* value. (Abbr.) – Abbreviation; (ABA) – Alkaloid-based analogue; (FRCD) – Furan ring-containing derivative.